Pesticide effects on German amphibians and consequences for their risk assessment in the European Union

Pestizideffekte auf deutsche Amphibien und Folgerungen für deren Risikobewertung in der Europäischen Union

by
Elena Adams
from Adenau, Germany

Accepted dissertation thesis for the partial fulfilment of the requirements for a
Doctor of Natural Sciences

Faculty 7: Natural and Environmental Sciences
University of Koblenz-Landau

Thesis examiners:
Dr. habil. Carsten A. Brühl, University of Koblenz-Landau
Prof. Dr. Ralf Schulz, University of Koblenz-Landau

Date of oral examination: June 25th, 2021

LIST OF PUBLICATIONS

The present thesis is a cumulative dissertation based on the following scientific publications:

I. **Appendix A.1:** Adams, E., Leeb, C., Roodt, A.P., Brühl, C.A. (2021). Interspecific sensitivity of European amphibians towards two pesticides and comparison to standard test species. Environmental Sciences Europe 33:49. DOI: 10.1186/s12302-021-00491-1

II. **Appendix A.2:** 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. DOI: 10.1016/j.scitotenv.2021.147865

III. **Appendix A.3:** Adams, E., Gerstle, V., Brühl, C.A. (2021). Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*). Environmental Toxicolology and Chemistry 50(5), 1289-1297. DOI: 10.1002/etc.4972

IV. **Appendix A.4:** Adams, E., Leeb, C., Brühl, C.A. (2021). Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape. Ecotoxicology 30, 213-223. DOI: 0.1007/s10646-020-02335-9

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Dr. habil. Carsten A. Brühl for his outstanding support and the valuable cooperation over many years of our collaboration. Many thanks to Prof. Dr. Ralf Schulz for examining this thesis and Jun.-Prof. Dr. habil. Mirco Bundschuh for charing the dissertation committee.

Many thanks to my colleagues which became friends over the last couple of years. Thank you, Tobias Schmitt, for laughing, singing and crying with me throughout many days and nights. Simon Lüderwald, Stefanie Allgeier, Christoph Leeb, Marco Konschak, and Philipp Uhl, thank you for valuable scientific discussions, your moral support, and revealing the joy of Aperol Spritz. Thanks Sara Goncalves, Nina Röder, Dominic Englert, Alexander Feckler, Verena Gerstle, Sebastian Pietz, and all others that I have forgotten. Thank you all for your support, an unforgettable time and being an awesome team.

My sincere thanks to Therese Bürgi who kept running the lab. You are sadly missed!

Several students have participated in my research. My thanks to Sara Kolbenschlag, Laura Schuler, Teresa Fagundes, Lars Bleidt, Robert Pleitner, Haiying Xia, and Ashvini Jonathan.

I also wish to express sincere thanks to my colleagues of the terrestrial vertebrates expert team of Bayer AG Division Crop Science for their unfailing support throughout the last months and valuable comments on my research and this thesis.

Further, I would like to express my sincere thanks to my best friends for sticking with me through all the ups and downs and bringing joy to my life. Thank you Luisa Schlüter, Bianca Frombold, and Judith May.

Many thanks to my brother, my parents and grandparents for their continuous support throughout my life.

Finally, I want to thank my significant other, Stephan – always – Müller. You make life better.

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ABBREVIATION LIST

AMA	Amphibian metamorphosis assay
a.s.	Active substance
BCF	Bioconcentration factor
CI	Confidence interval
DESTATIS	Statistisches Bundesamt
DT50	Dissipation time 50%
E.C.	Emulsifiable concentrate
EC	Effect concentration
EFSA	European Food Safety Authority
ERA	Environmental risk assessment
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
FR	Field rate
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GS	Gosner stage
HC5	Hazard concentration 5%
IUCN	International Union for Conservation of Nature
IVA	Industrieverband Agrar
K_{OC}	Soil adsorption coefficient
K_{OW}	*n*-octanol-water partition coefficient
LC50	Lethal concentration 50%

LD50	Lethal dose 50%
LL50	Lethal loading rate 50%
n.a.	Not applicable
NOEC	No observed effect concentration
MDR	Model deviation ratio
OECD	Organisation for Economic Co-operation and Development
PEC	Predicted environmental concentration
PPDB	Pesticide Property DataBase
RAC	Regulatory acceptable concentration
RQ	Research question
SC	Suspension concentrate
SSD	Species sensitivity distribution
SumTU	Sum of toxic units
TER	Toxicity exposure ratio
U.S. EPA	United States Environmental Protection Agency
WDG	Water dispersible granule

ABSTRACT

Vertebrate biodiversity is rapidly decreasing worldwide with amphibians being the most endangered vertebrate group. In the EU, 21 of 89 amphibian species are recognized as being endangered. The intensively used European agricultural landscape is one of the major causes for these declines. As agriculture represents an essential habitat for amphibians, exposure to pesticides can have adverse effects on amphibian populations. Currently, the European risk assessment of pesticides for vertebrates requires specific approaches for fish regarding aquatic vertebrate toxicity and birds as well as mammals for terrestrial vertebrate toxicity but does not address the unique characteristics of amphibians. Therefore, the overall goal of this thesis was to investigate the ecotoxicological effects of pesticides on Central European anuran amphibians. For this, effects on aquatic and terrestrial amphibian life stages as well as on reproduction were investigated. Then, in anticipation of a risk assessment of pesticides for amphibians, this thesis discussed potential regulatory risk assessment approaches.

For the investigated pesticides and amphibian species, it was observed that the acute aquatic toxicity of pesticides can be addressed using the existing aquatic risk assessment approach based on fish toxicity data. However, lethal as well as sublethal effects were observed in terrestrial juveniles after dermal exposure to environmentally realistic pesticide concentrations, which cannot be covered using an existing risk assessment approach. Therefore, pesticides should also be evaluated for potential terrestrial toxicity using risk assessment tools before approval. Additionally, effects of co-formulants and adjuvants of pesticides need specific consideration in a future risk assessment as they can increase toxicity of pesticides to aquatic and terrestrial amphibian stages. The chronic duration of combined aquatic and terrestrial exposure was shown to affect amphibian reproduction. Currently, such effects cannot be captured by the existing risk assessment as data involving field scenarios analysing effects of multiple pesticides on amphibian reproduction are too rare to allow comparison to data of other terrestrial vertebrates such as birds and mammals. In the light of these findings, future research should not only address acute and lethal effects, but also chronic and sublethal effects on a population level. As pesticide exposure can adversely affect amphibian populations, their application should be considered even more carefully to avoid further amphibian declines. Overall, this thesis emphasizes the urgent need for a protective pesticide risk assessment for amphibians to preserve and promote stable amphibian populations in agricultural landscapes.

ZUSAMMENFASSUNG

Die Biodiversität von Vertebraten nimmt weltweit rapide ab, wobei Amphibien die am stärksten gefährdete Wirbeltiergruppe darstellen. In der EU sind 21 von 89 Amphibienarten bedroht. Die intensiv genutzte europäische Agrarlandschaft ist eine der Hauptursachen für diese Rückgänge. Da die Agrarlandschaft einen bedeutenden Lebensraum für Amphibien darstellt, kann die Exposition zu Pestiziden negative Auswirkungen auf Amphibienpopulationen haben. Derzeit erfordert die europäischen Risikobewertung von Pestiziden für Vertebraten spezifische Ansätze für Fische hinsichtlich der aquatischen Vertebratentoxizität und für Vögel sowie Säugetiere in Bezug auf die terrestrische Vertebratentoxizität. Die besonderen Eigenschaften von Amphibien werden jedoch nicht berücksichtigt. Daher war das übergeordnete Ziel dieser Arbeit, die ökotoxikologischen Effekte von Pestiziden auf mitteleuropäische Froschlurche zu untersuchen. Dazu wurden Effekte auf aquatische und terrestrische Amphibienstadien sowie auf deren Reproduktion untersucht. Anschließend wurden in dieser Arbeit in Erwartung einer Risikobewertung von Pestiziden für Amphibien mögliche regulatorische Risikobewertungsansätze diskutiert.

Für die untersuchten Pestizide und Amphibienarten wurde festgestellt, dass die akute aquatische Toxizität von Pestiziden mit dem bestehenden Ansatz der aquatischen Risikobewertung auf der Grundlage von Fischtoxizitätsdaten abgedeckt werden kann. Jedoch wurden bei terrestrischen Juvenilen nach dermaler Exposition zu umweltrealistischen Pestizidkonzentrationen sowohl letale als auch subletale Effekte beobachtet, die mit keinem verfügbaren Risikobewertungsansatz erfasst werden können. Daher sollten Pestizide vor der Zulassung auch auf eine potenzielle terrestrische Toxizität mit Hilfe von Risikobewertungsinstrumenten geprüft werden. Darüber hinaus müssen die Auswirkungen von Bei- und Hilfsstoffen von Pestiziden bei einer zukünftigen Risikobewertung besonders berücksichtigt werden, da sie die Toxizität von Pestiziden gegenüber aquatischen und terrestrischen Amphibienstadien erhöhen können.

Des Weiteren wurde gezeigt, dass die chronische Dauer einer kombinierten aquatischen und terrestrischen Exposition die Reproduktion von Amphibien negativ beeinflusst. Gegenwärtig können solche Effekte von der bestehenden Risikobewertung nicht erfasst werden, da Daten aus Feldszenarien, die die Auswirkungen mehrerer Pestizide auf die Reproduktion von Amphibien abbilden, zu selten sind, um einen Vergleich mit Daten anderer terrestrischer Wirbeltiere wie Vögel und Säugetiere zu ermöglichen. In Anbetracht dieser Erkenntnisse sollten sich zukünftige Untersuchungen nicht nur mit akuten und letalen Effekten, sondern auch mit chronischen und subletalen Effekten auf Populationsebene befassen. Da sich die Exposition gegenüber Pestiziden negativ auf Amphibienpopulationen auswirken kann, sollte ihr Einsatz noch sorgfältiger überlegt werden, um einen weiteren Rückgang der Amphibien zu vermeiden. Insgesamt unterstreicht diese Arbeit die dringende Notwendigkeit einer protektiven Pestizidrisikobewertung für Amphibien, um Amphibienpopulationen in Agrarlandschaften zu erhalten und zu fördern.

1 INTRODUCTION

1.1 Ecology and biology of European amphibians

Amphibians are cold-blooded vertebrates and include more than 7000 known species. They are grouped in the three orders Caudata (~680 newt and salamander species), Anura (~6500 toad and frog species) and Gymnophiona (~200 caecilian species), the latter being absent from Europe (Stuart, 2008). In Europe, there are 89 native amphibian species (53 anurans and 36 caudates; Sillero et al., 2014), of which 20 species are native to Germany (14 anurans and 6 caudates; Rote-Liste-Gremium Amphibien und Reptilien, 2020).

With a few exceptions, temperate amphibians have a biphasic life cycle inhabiting both aquatic and terrestrial habitats. Outside of their breeding season they live in terrestrial habitats for hibernation and foraging. In temperate and cold-temperate regions, the breeding season of amphibians begins in spring. At this time, adult amphibians migrate from their refuges to their breeding ponds which can be located up to several kilometres away from their terrestrial habitats (Günther, 2009). The reproductive cycle of most temperate amphibian species involves an aquatic egg in which an embryo develops to an aquatic larva. Until summer or autumn, these tadpoles undergo a metamorphosis during which they transform into terrestrial, air-breathing juveniles, which is morphologically and anatomically similar to an adult and moves to the terrestrial habitat. At sexual maturity, this migration is repeated every year.

Due to the above described biphasic life cycle, amphibians are considered as an indicator species of general environmental health (Collins and Storfer, 2003). One of the many key characteristics of amphibians is their poikilothermy. This temperature-dependency determines many aspects of amphibian physiology such as their metabolic rate, oxygen consumption and energy expenditure (Stuart, 2008). Their thermoregulation is controlled by both behavioural and physiological mechanisms such as movement to warmer or cooler sites or changing evaporative cooling through their skin (Stuart, 2008). This permeable skin is another unique characteristic making amphibians highly sensitive to their environment and water balance a critical issue. Skin is the main route of both water uptake and loss in amphibians and thus facilitates diffusion of water but also chemical agents (Quaranta et al., 2009). Next to its function for water regulation, the skin is also an important respiratory organ in amphibians. Especially for juveniles with a high surface-to-volume ratio, skin breathing covers an essential part of respiration (up to 30% of O_2 uptake and 70% CO_2 elimination; Burggren and Moallf, 1984).

1.2 Amphibian population decline

Vertebrate biodiversity is rapidly decreasing worldwide with amphibians considered the most endangered vertebrate group (Hoffmann et al., 2010). Latest reports of the International Union for the Conservation of Nature (IUCN) suggest that 41% of all amphibian species are threatened (IUCN, 2020). In the EU, 21 of 89 amphibian species are recognized as endangered i.e. listed within the IUCN categories of critically endangered, endangered, or vulnerable for their global conservation status. This ratio can be even worse in some locations, when national or regional red lists are considered. According to the German Red List categories, German amphibians are largely endangered, very vulnerable or vulnerable, or near threatened (Rote-Liste-Gremium Amphibien und Reptilien, 2020). From 2000 to 2018, 15 out of 20 native amphibian species showed a short-term trend of declining populations. Additionally, 17 species showed a long-term decline in the last 50 to 150 years (Rote-Liste-Gremium Amphibien und Reptilien, 2020). Furthermore, most species are listed as endangered in at least one federal state of Germany (Rote-Liste-Gremium Amphibien und Reptilien, 2020). Exposure to anthropogenic pollutants such as agrochemicals is hypothesized to be one of the main causes of amphibian decline (Stuart et al., 2004). Other important stressors are habitat loss and fragmentation, invasive species, diseases, climate change and over-exploitation (Collins and Storfer, 2003) as well as interaction of these stressors which can cause much more severe effects (e.g. Relyea, 2003).

1.3 Pesticide exposure to amphibians in agricultural landscapes

In 2019, 50.7% of the German land coverage was used for agriculture, making this area one of the largest terrestrial biomes in Germany (Destatis, 2020). About 90% of the agricultural land is cropped and managed with associated modern measures such as the use of pesticides (IVA, 2021). All German anuran species can be found in open agricultural landscapes as well as in rich structured agricultural landscapes consisting of a mosaic of arable fields, forests and grasslands (Berger et al., 2011). Some species such as the spadefoot toad and the crested newt even prefer agricultural fields over off-field habitats (Berger et al., 2011; Cooke, 1986).

Considerable amounts of pesticides are applied to agricultural landscapes. Currently, 466 substances are registered for use in the European Union (European Commission, 2021). Worldwide, four million tons of pesticide active substances were used in or sold to the agricultural sector in 2018. In Europe, 478000 tons were used with 45000 tons specifically in Germany (FAO, 2021). Pesticides are applied to reduce pest pressure (e.g. insects, fungal

diseases, or weeds), but they can also have inadvertent effects on non-target species such as amphibians. In general, agricultural land-use and corresponding agricultural activities such as the use of pesticides are highly correlated with global amphibian population declines (Houlahan and Findlay, 2003). In Germany, pesticides are considered a major reason for declining amphibian populations (Berger et al., 2011; Günther, 2009; Rote-Liste-Gremium Amphibien und Reptilien, 2020). Due to their biphasic life cycle amphibians can be exposed to pesticides both in their aquatic and in their terrestrial habitat. Because amphibians often breed in water bodies in agricultural landscapes such as rain retention ponds they can be exposed to pesticides occurring in such waters (Souza et al., 2020) as adults and during their larval development. Furthermore, they move as metamorphosed juveniles or adults through these agricultural landscapes (Berger et al., 2013; Lenhardt et al., 2015). Due to the spatial and temporal overlap of pesticide applications and amphibian migrations (Lenhardt et al., 2015), amphibians can be exposed dermally to pesticide contaminated soil, vegetation and orally *via* food caught in their terrestrial habitat. Although amphibians on land are mostly nocturnally active, activity during day time may occur so that a direct pesticide overspray cannot be excluded (Leeb et al., 2020a). Additionally, some pesticides are only applied at night to avoid direct bee exposure. Therefore, migrating amphibians can also be oversprayed during night.

The level of terrestrial exposure to adults and juveniles depends on several variables. In case of pesticide spray applications, not only the pesticide type, frequency, and amount of applications play an important role but also the season of pesticide use and therefore the presence of crops in-field and their role as potential canopy cover (interception). Such cover can reduce the exposure significantly (Cusaac et al., 2017). Depending on the activity phase of the amphibian species, particularly adults and juveniles leaving the breeding ponds may face high pesticide exposure levels. This can vary depending on the type of pesticide applied and relevant interception values. For example, low interception by crops leads to high herbicide exposure in maize fields (Berger et al., 2013). In addition to in-field exposure, amphibians can also be exposed in neighbouring non-crop areas *via* run-off and spray-drift during pesticide applications.

1.4 European risk assessment of pesticides for vertebrates and limitations for amphibians

Until 2009, the “Council Directive 91/414/EEC of July 1991 concerning the admission of plant protection products on the market”, regulated the use of pesticides in the European Union (EU). According to this directive, vertebrate ecotoxicology of fish for the aquatic environment as well

as birds and mammals for the terrestrial environment had to be considered in the environmental risk assessment (ERA) of pesticides whereas amphibian ecotoxicology was not considered specifically. Since 2009, this directive was replaced by the "Regulation (EC) No 1107/2009 of the European Parliament and the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC". This regulation states that pesticides (active substances and formulated pesticide products) are only introduced to the market if they *"do not have any harmful effect on human or animal health or any unacceptable effects on the environment"* (European Commission, 2009). Furthermore, they shall have no unacceptable effects on non-target species, ecosystems, and biodiversity (European Commission, 2009). This infers that the risk for amphibians should be addressed as part of the ecotoxicological risk assessment for pesticides.

In the EU, the current authorisation process for pesticides follows a 2-tiered approach: First, a pesticide active substance needs to be approved by the EU (European Food Safety Authority, EFSA). Then plant protection products, also known as formulations, which include the approved active substance, should be reviewed for approval by national authorities of the individual member states. For risk assessment purposes, it is important to note that pesticide formulations are mixtures of one or more active substances and co-formulants (European Commission, 2009). Following EU terminology, co-formulants are substances or preparations which are used in pesticide formulations or adjuvants (European Commission, 2009). They are neither active substances nor synergists or safeners, which enhance the activity of the active substance or reduce phytotoxic effects of pesticides on certain plants, respectively. Co-formulants that have been proven to *"have a harmful effect on human or animal health […] or an unacceptable effect on the environment*" shall not be accepted for inclusion in pesticide formulations (European Commission, 2009). Adjuvants are substances or preparations consisting of such co-formulants and are placed on the market separately to be mixed with a pesticide formulation before application to enhance efficacy or applicability (European Commission, 2009). Thus, co-formulants and adjuvants are introduced together with active substances to the environment, potentially exerting additional adverse effects.

Current risk assessment approaches for vertebrates are based on a toxicity exposure ratio (TER) assessment which is calculated by dividing a toxicity endpoint such as an effect or lethal concentration (EC or LC) or a no effect concentration (NOEC) by the exposure e.g. a predicted environmental concentration (PEC). There are no specific test guidelines or risk assessment approaches to assess pesticide toxicity to amphibians. The risk assessment of potential effects

on aquatic amphibian life stages is assumed to be covered by standard test organisms such as fish (Ockleford et al., 2018). The aquatic risk assessment approach for fish is a tiered approach described in a guidance document of EFSA (EFSA, 2013). In principle, the exposure is assessed using FOCUS (2001) exposure models to calculate PECs for surface waters. To determine effect concentrations such as a LC50 for acute toxicity or a NOEC for chronic toxicity, ecotoxicological tests are performed with standard fish species. For every substance, acute toxicity testing is required for rainbow trout (*Oncorhynchus mykiss*; EFSA, 2013). Chronic toxicity tests have to be performed if continued or repeated exposure is likely and if the acute TER is below the safety trigger of 100. The derived endpoints are divided by an assessment (safety) factor of 100 for acute toxicity studies and 10 for chronic toxicity studies to determine a regulatory acceptable concentration (RAC). For risk assessment, the RAC is compared to the maximum PEC for acute risk assessment and to a time-weighted average PEC for the chronic risk assessment. If the PEC is lower than the RAC, the risk is assumed to be acceptable. If an unacceptable risk is projected, a higher tier risk assessment with a refined exposure assessment (refined FOCUS model), a refined effect assessment (e.g. tests with model ecosystems, which lead to a lower assessment factor and/or higher endpoint value), and risk mitigation measures (e.g. suitable application techniques, no-spray zones, temporal or spatial restrictions of use) can be performed.

To guarantee the coverage of amphibians by fish, it is important to assess the sensitivity of amphibians to pesticides and compare their sensitivity to other taxa such as the standard fish test species *O. mykiss*. Weltje et al. (2013) compared acute and chronic toxicity data of fish and amphibians and observed that amphibians were 10 to 100 times more sensitive to four out of 55 (acute toxicity) and two out of 52 (chronic toxicity) pesticides. Because fish were more sensitive for most of the investigated pesticides, Weltje et al. (2013) argue that fish sensitivities are appropriate to cover the sensitivity of aquatic amphibian stages and that additional amphibian testing is not necessary. However, the majority of these comparisons is based on pesticides that are no longer commonly used in the EU (e.g. DDT, atrazine, carbaryl or chlorinated pesticides like chlorpyrifos and lindane). In addition, many of these studies focus on amphibian species not native to Europe such as North American species or (sub-) tropical species like the African clawed frog (*Xenopus laevis*). *Xenopus laevis* is often used as model species for amphibians (Aldrich, 2009; Hoke and Ankley, 2005) because it is easy to culture and handle in laboratory and there is a wide knowledge of its developmental biology (Deuchar, 1972). However, there are few comparative toxicity data for *X. laevis* relative to other

amphibian species. Several studies have found that *X. laevis* is more tolerant to environmental pollutants than native amphibian species (Birge et al., 1985; Hoke and Ankley, 2005; Ortiz-Santaliestra et al., 2018). For example, the European common frog (*Rana temporaria*) was described as more sensitive than *O. mykiss* and *X. laevis* towards heavy metals and industrial effluents (Birge et al., 1985). It remained unclear whether the comparison of fish data to the sensitivity of tadpoles of the completely aquatic living species *X. laevis* is also protective for semi-aquatic species native in Europe (Ockleford et al., 2018).

Similar to the aquatic risk assessment, the terrestrial risk assessment of birds and mammals is also based on a tiered approach (EFSA, 2009b): The first screening step of so-called indicator species is performed with worst-case assumptions regarding their exposure in order to highlight substances that do not require further consideration due to low risk. The second step is a first-tier assessment for acute and reproductive risks applying more realistic exposure estimates with generic focal species, which are assumed to represent real species occurring in a particular environment. Focal species are selected assuming their exposure would be greatest. Therefore, if their risk is considered acceptable, it is assumed to be protective for all species they represent in that landscape. Higher tier approaches are needed if the acceptability criteria for the calculated TER are not met. This higher tier assessment provides a greater degree of realism with diverse exposure estimates such as interception by crops and behaviour data of real focal species to calculate the risk.

Toxicity testing of birds and mammals is generally based on oral uptake of food, soil and water. Thus, only dietary exposure is included in the risk assessment for terrestrial vertebrates. For terrestrial amphibian life stages, there are two relevant exposure pathways for the exposure to pesticides. On the one hand, juveniles and adults might feed on contaminated arthropods. Crane et al. (2016) compared the single-dose oral toxicity of 26 chemicals including 18 pesticides between amphibians and birds as well as mammals. Birds and mammals were more sensitive to these chemicals than the amphibians except for DDT. Therefore, Crane et al. (2016) argue that the oral toxicity of pesticides to amphibians is covered by the current risk assessment procedures for birds and mammals. On the other hand, the highly permeable and sensitive skin of amphibians results in a two times faster uptake of chemicals in comparison to mammals (Quaranta et al., 2009; van Meter et al., 2014). This is why dermal uptake is the most relevant route of exposure for terrestrial amphibian stages that cannot be covered by the risk assessment of birds and mammals.

Weltje et al. (2017; 2018) collected and reviewed available acute dermal toxicity data on anurans to develop a semi-quantitative calculation tool for dermal toxicity risk assessment (s. chapter 3.4). This tool is suggested as a screening step to distinguish pesticides of concern from those of no concern. However, it only covers dermal exposure to active substances. Several studies indicate that co-formulants in products can be toxic themselves or enhance the toxicity of pesticide formulations to amphibians (e.g. Brühl et al., 2013; Hooser et al., 2012; Wagner et al., 2015). Amphibians might be exposed to co-formulants and adjuvants because of their biphasic life-cycle and so experience a combined aquatic and terrestrial exposure. Furthermore, their permeable skin that enables water regulation (Wells, 2007) also facilitates the uptake of larger molecules such as pesticides through the dermal barrier which makes them also highly sensitive to dermal exposure to pesticide formulations (Kaufmann and Dohmen, 2016; Quaranta et al., 2009). Several studies observed increased dermal absorption of pesticide formulations and co-formulants in comparison to their active substances alone (Baynes and Riviere, 1998; Brand and Mueller, 2002; Reifenrath, 2007). Therefore, amphibians are especially vulnerable due to their high dermal uptake capacity.

In addition to enhanced absorption, increased toxicity of formulations or toxicity of co-formulants themselves were observed in several studies investigating lethal and sublethal level effects in amphibians. Increased mortality of aquatic stages after exposure to formulations of the insecticide permethrin (Boone, 2008) and the herbicide glyphosate (Howe et al., 2004) has been observed in comparison to the active substances alone. Lethal effects of a fungicidal pyraclostrobin formulation and a herbicidal glyphosate formulation have been shown for early terrestrial amphibian stages (Brühl et al., 2013; Relyea, 2005). Effects of formulations were also detected on a sublethal level such as effects on the aquatic development after exposure to glyphosate formulations (Howe et al., 2004) and *in vitro* neurotoxic effects after exposure to organophosphorous insecticide formulations (Swann et al., 1996). These studies show that knowledge about the toxicity of active substances does not *per se* allow a prediction about the effect of pesticide formulations. Another unique characteristic of amphibians that is not covered appropriately by the current vertebrate risk assessment and the suggested tool of Weltje et al. (2017) is the chronic aquatic and terrestrial exposure of amphibians and the effect of this combined exposure on their reproduction. Only a few studies are available which investigated the effects of pesticides on the reproduction of amphibians (e.g. Bókony et al., 2018; Hayes et al., 2010; Moore, 1983). A chronic, sublethal exposure to pesticides might therefore lead to further amphibian population declines due to an impaired reproductive performance.

2 OBJECTIVES AND THESIS OUTLINE

The overall goal of this thesis was to contribute to the identification of ecotoxicological effects of pesticides on anuran amphibians. Then, in anticipation of a risk assessment of pesticides for amphibians, this thesis discussed potential regulatory risk assessment approaches for aquatic and terrestrial stages of Central European amphibian species. Therefore, this thesis investigated four research questions (RQ), each presented in a separate scientific publication. After the summary and general discussion of findings, implications for future risk assessment procedures for aquatic and terrestrial amphibian stages and a general outlook will be given. Figure 1 provides a conceptual overview of this thesis, the corresponding research questions, and reference to the authors´ contributions of scientific publications (Appendix A.1-A.4).

RQ-1 (Publication 1 – Appendix A.1) of the present thesis comprised the question, whether aquatic standard test species such as *Oncorhynchus mykiss* or commonly used model laboratory organisms such as *Xenopus laevis* might be protective surrogates also for Central European amphibian species? Therefore, the aim of RQ-1 was to assess the sensitivity of larvae of eight native Central European species to commercial formulations of the two pesticides folpet and indoxacarb. In addition, the sensitivity of these native species was compared to the sensitivity of *X. laevis* using experimentally derived sensitivities of *X. laevis*, and to the sensitivity of *O. mykiss* using regulatory endpoint values from literature.

In the second research question RQ-2 (Publication 2 – Appendix A.2) it was investigated how co-formulants and adjuvants of pesticide formulations affect the sensitivity of European common frogs (*Rana temporaria*). Thus, the aims of RQ-2 were to analyse the aquatic toxicity differences between the herbicide formulation Focus® Ultra, its active substance cycloxydim, its two co-formulants solvent naphtha and docusate as well as the adjuvant Dash® E.C, that is part of the combination package Focus® Aktiv-Pack. The experimentally determined formulation and package toxicities were compared to predicted toxicity values based on a concentration addition model. In addition, lethal and sublethal effects including effects on body mass and locomotor activity of environmentally relevant concentrations of each substance on terrestrial juveniles were investigated.

RQ-3 (Publication 3 – Appendix A.3) investigated whether an environmentally realistic dermal pesticide exposure of viticultural fungicides affects juvenile terrestrial stages of amphibians. The objectives of RQ-3 were to investigate sublethal effects, including effects on body mass,

locomotor activity, as well as feeding behaviour, and to evaluate the potential for delayed influence of pesticide exposure during the previous aquatic larval development on the terrestrial sensitivity of juvenile *R. temporaria*. Furthermore, sensitivity differences to two fungicide formulations with the same active substance were analysed to analyse formulation effects. For the purpose of this thesis, the terrestrial risk assessment for the fungicide was performed (unpublished data) according to the suggested screening tool of Weltje et al. (2018).

RQ-4 (Publication 4 – Appendix A.4) covered the question whether the combined chronic aquatic and terrestrial exposure to pesticides affect amphibian reproduction. To address this question, the reproductive capacity of common toads (*Bufo bufo*) in the viticultural landscape of Palatinate in Southwest Germany was investigated along a pesticide gradient by means of fecundity, fertilization rate as well as offspring survival and size.

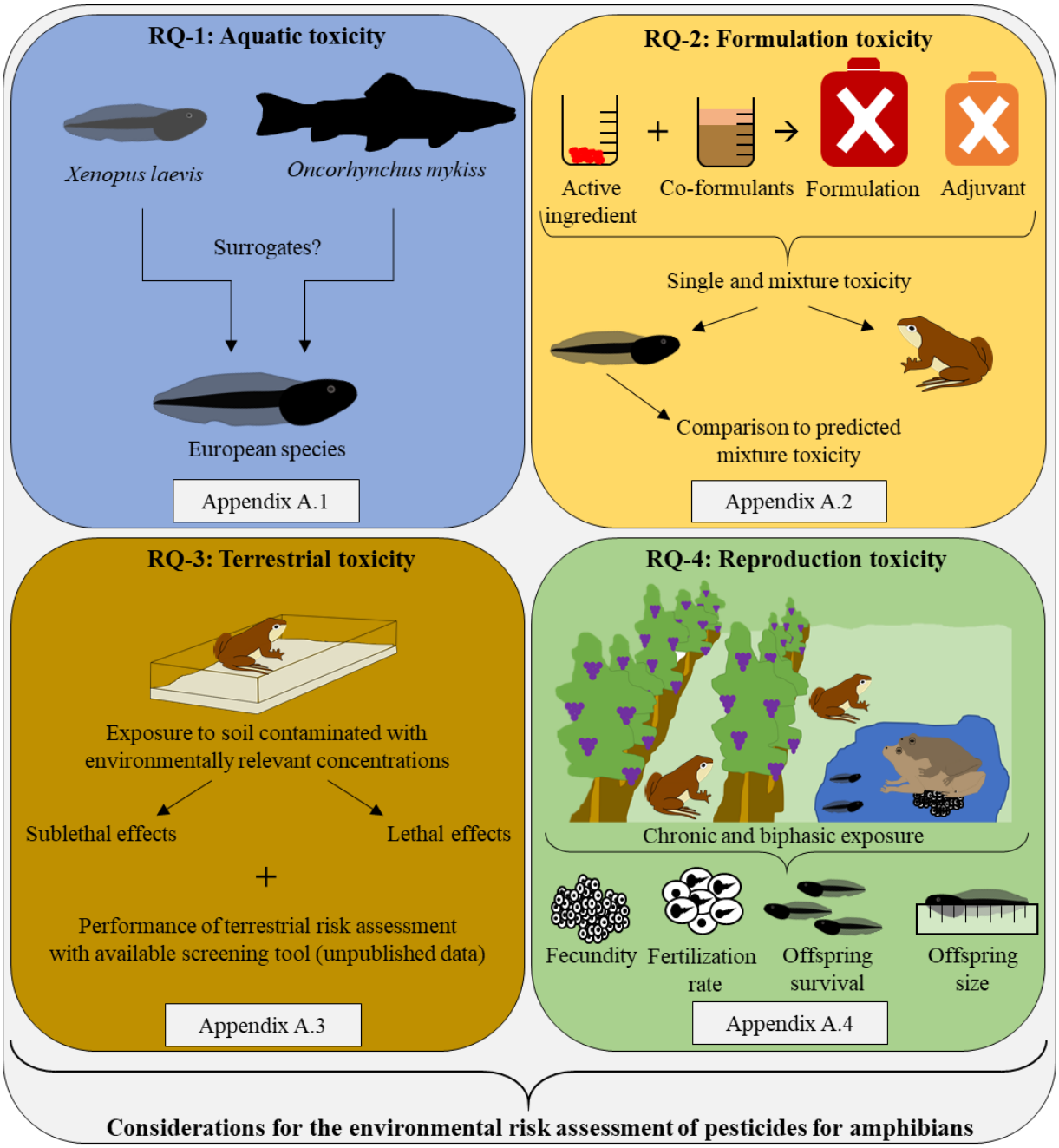

Figure 1. Conceptual overview of the four main research questions addressed in this thesis (RQ1 – RQ4) and their corresponding scientific publications (Appendix A.1 – A.4).

3 METHODOLOGICAL OVERVIEW

In this thesis, three main approaches have been used to assess the ecotoxicological effects of pesticides on amphibians ranging from acute aquatic laboratory studies (RQ-1 and RQ-2) over acute terrestrial laboratory studies (RQ-2 and RQ-3) to a semi-field study (RQ-4).

3.1 Animal collection and husbandry for laboratory studies

In total, nine different amphibian species were investigated in at least one of the studies performed for the present thesis (Table 1).

Table 1. Studied amphibian species and corresponding research questions.

Species	Common name	Research question (RQ)
Rana temporaria	Common frog	1, 2, 3
Bufo bufo	Common toad	1, 4
Hyla arborea	Common tree frog	1
Rana dalmatina	Agile frog	1
Bufotes viridis	European green toad	1
Pelobates fuscus	Common spadefoot toad	1
Pelophylax sp.	Pool frog, Edible frog or hybrid of both	1
Epidalea calamita	Natterjack toad	1
Xenopus laevis	African clawed frog	1

Native species were collected as embryos or early hatchlings from several breeding ponds in South Germany. *Xenopus laevis* embryos were obtained from an in-house culture of Eurofins Agroscience Services EcoChem GmbH (Niefern-Öschelbronn, Germany). Tadpoles were reared either in aquaria in a climate chamber under controlled laboratory conditions (RQ-1, RQ-2) or in net cages in eight ponds (Figure 2) in the winegrowing region Südliche Weinstraße and the Palatinate forest (RQ-3). Developmental stages were assigned according to Gosner (1960) for native species and Nieuwkoop and Faber (1994) for *X. laevis*. Metamorphosed juveniles of RQ-3 were kept in outdoor cages filled with forest soil, moss, leaves, and a water supply, under natural conditions, placed outside of the research station Geilweilerhof of the University of Koblenz-Landau. Terrestrial juveniles investigated in RQ-2 were kept in terraria filled with moisturized forest soil, moss, leaves and a water supply in a climate chamber under controlled

conditions. Until test initiation, all terrestrial juveniles were fed ad libitum with *Drosophila melanogaster* and *D. hydei* obtained from an in-house culture.

3.2 Study pond toxicity

To quantify the pesticide concentration gradient in the eight rearing ponds investigated in RQ-3 including the five breeding ponds of RQ-4, five grab water samples from each pond were analysed for 58 pesticides (Figure 2, Appendix A.3). The pond toxicity was assessed according to the sum of toxic units approach (SumTU, Schäfer et al., 2011) using data of acute fish toxicity studies compiled from the Pesticide Properties DataBase (PPDB; Agriculture and Environment Research Unit of the University of Hertfordshire, 2013) as proxy for amphibians (Weltje et al., 2013). Furthermore, the landscape composition around the study ponds was analysed to estimate the terrestrial exposure to individuals of RQ-4 (Table 2).

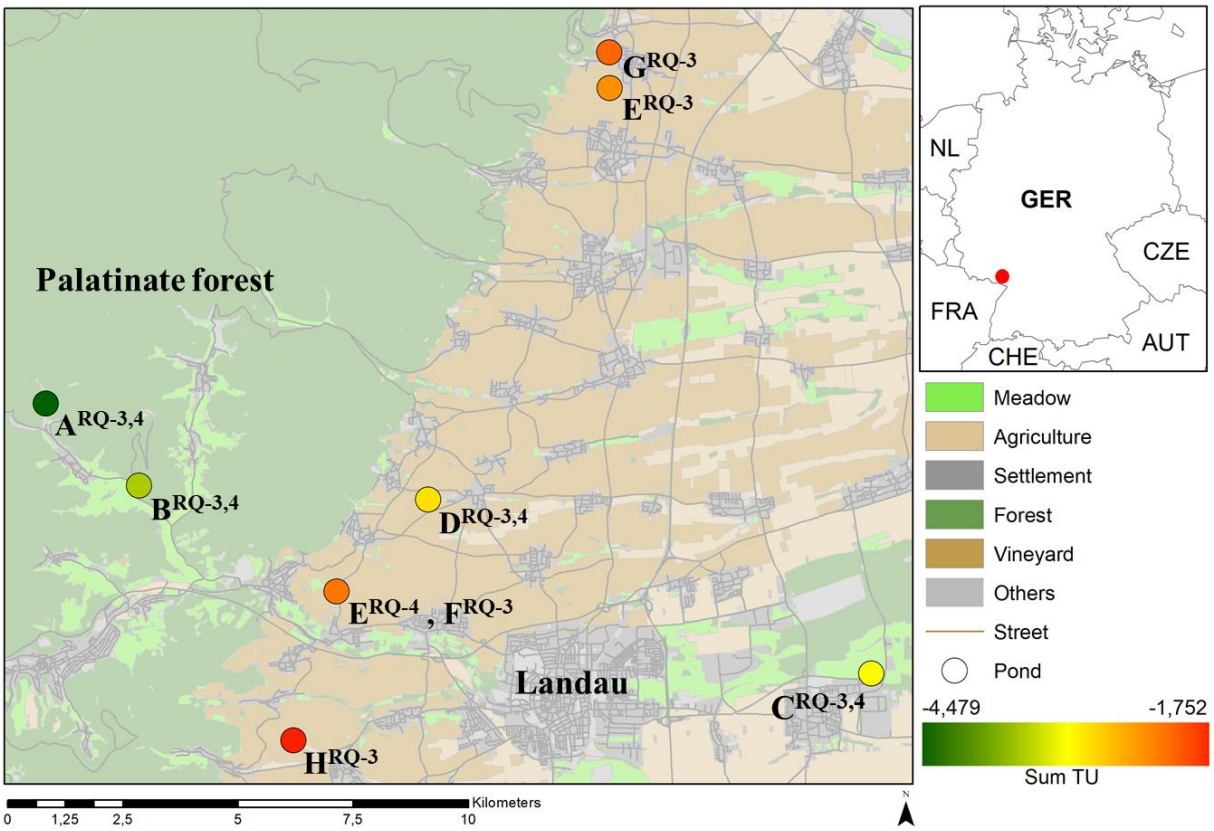

Figure 2. Map of study ponds investigated in RQ-3 and RQ-4 in Palatinate in Southwest Germany. Increasing letters and colours of study sites represent the pesticide contamination (SumTU) from no contamination (dark-green) to high contamination (red). Superscripts of pond designation represent pond designation depending on the investigation in RQ-3 or RQ-4. **[Modified from Appendix A.3 and Appendix A.4]**

Table 2. Landscape composition in a radius of 3000 m around the study ponds investigated for RQ-4 based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50). **[Appendix A.4]**

Pond	Viticulture [%]	Other agriculture [%]	Meadow [%]	Settlement [%]	Forest [%]	Other [%]
A	0.0	0.0	5.1	1.3	92.9	0.6
B	0.1	1.1	19.2	5.6	72.1	1.9
C	0.3	31.4	19.6	15.5	28.5	4.8
D	47.5	1.1	7.9	11.6	29.8	2.2
E	57.0	3.1	6.1	10.1	22.5	1.3

3.3 Acute aquatic toxicity tests

For RQ-1, aquatic acute toxicity tests with the fungicidal formulation Folpan© 500 SC (a.s. folpet, hereafter Folpan SC) and the insecticidal formulation Avaunt© E.C. (a.s. indoxacarb, hereafter Avaunt) were performed with eight German amphibian species and the laboratory model species *X. laevis* as 96-h dose-response tests. The identified LC50 values of the native species were used to generate a species sensitivity distribution (SSD) for each pesticide formulation to allow species comparison and to derive the 5% hazard concentration (HC5). Afterwards, the native amphibian sensitivities were compared to the sensitivity of *X. laevis* and to literature values of *O. mykiss* (Adama, 2015; Cheminova, 2020). Furthermore, RACs based on the fish sensitivities were compared to the calculated HC5 values derived from the native amphibian SSDs.

In RQ-2, similar 96-h dose-response tests were performed with tadpoles of *R. temporaria*. To assess formulation toxicity, acute toxicity tests were performed with the herbicide formulation Focus® Ultra (hereafter Focus), its active substance cycloxydim, its two co-formulants solvent naphtha and docusate as well as the adjuvant Dash® E.C (hereafter Dash) that is part of the combination package Focus® Aktiv-Pack. To determine whether formulation and tank mixture (formulation + adjuvant) toxicities to amphibians can be predicted based on the single compound toxicity and the relative fraction of each mixture component, the predicted aquatic mixture toxicities for the combination of cycloxydim, solvent naphtha and docusate as well as for the combination of Focus and Dash were calculated using a concentration addition model according to Kortekamp et al. (2011). The model deviation ratio (MDR) according to EFSA (2013) was calculated to counter-check the measured mixture toxicity of the formulation as

well as the combination of the formulation and the adjuvant and to determine if the components react more (i.e. synergistically) or less (i.e. antagonistically) than expected.

3.4 Acute terrestrial toxicity tests

For RQ-2, terrestrial soil exposure tests were performed over 48 hours in a laboratory under controlled conditions. Freshly metamorphosed *R. temporaria* juveniles were kept individually in plastic terrariums filled with artificial soil. Each treatment group of twelve individuals was exposed to soil contaminated with cycloxydim, docusate, solvent naphtha, Focus, Dash, or Focus and Dash in combination. On the one hand, a worst-case scenario with the maximum recommended field rate was tested (100% FR). To increase the environmental relevance and to consider pesticide exposure mitigation by crop interception, a second test using 10% of the maximum recommended FR was performed. Cycloxydim was only tested for the 10% FR tests due to its limit of solubility in the 100% FR tests. Next to lethal effects after exposure to 100% of the FR, sublethal effects on the juveniles´ locomotor activity and body mass decline after 48 hours were investigated after exposure to 10% of the FR.

The terrestrial study performed for RQ-3 examined acute effects of the dermal exposure to two folpet formulations (Folpan® 80 WDG with 38-42% a.s. folpet and Folpan® 500 SC with 78-85% a.s. folpet, hereafter Folpan WDG and Folpan SC) on juvenile *R. temporaria* which were kept in ponds of different pesticide contamination (Figure 2) during their larval development. The setup was similar to the terrestrial studies of RQ-2. Half of the maximum recommended field rate (50% FR) of Folpan WDG was applied to the soil. To allow comparison of effects induced by both formulations, equal amounts of the a.s. were applied for Folpan SC. Next to lethal effects, sublethal effects on the juveniles´ locomotor activity, feeding behaviour, and body mass decline were investigated. To investigate, whether the suggested risk assessment tool of Weltje et al. (2018) would have indicated the high determined terrestrial amphibian toxicity of Folpan WDG, the terrestrial risk assessment was simulated for the purpose of this thesis as follows (unpublished data):

The tool is based on a correlation of acute fish lethal doses (LD50) and acute terrestrial amphibian LD50 values (Weltje et al., 2017). Using 96-h fish acute LC50 and fish bioconcentration factor (BCF) values, the fish lethal body burden (fish LD50) can be calculated according to equation 1. Where possible, this should be done with endpoints of the same fish species and with a steady-state BCF reached within 96 hours to allow direct species and temporal comparison.

$$LD50_{fish} = LC50_{fish} \times BCF_{fish} \quad (1)$$

Using the regression model derived by Weltje et al. (2017), a dermal amphibian LD50 can be estimated for an anuran exposed to dorsal overspray according to equation 2.

$$\log(LD50_{amphibian}) = 0.852 \times \log(LD50_{fish}) + 0.226 \quad (2)$$

To calculate the internal dose of a juvenile anuran after exposure to a specific application rate, equation 3 is used.

$$Dose = \frac{application\ rate \times dorsal\ area}{body\ weight} \quad (3)$$

with

dorsal area = assumed to be 3.04 cm² according to Weltje et al. (2018)

body weight = assumed to be 1.4 g according to Weltje et al. (2018) and U.S. EPA (2008)

To calculate a TER, the obtained Dose is divided by the $LD50_{amphibian}$ of the investigated pesticide. This value might be refined by means of crop interception, behavioural (such as hiding behaviour) or body weight data relevant for the time of application and is then compared to a trigger value of five. If the TER exceeds this value, the risk is assumed to be acceptable.

3.5 Semi-field study to investigate long-term effects on reproduction

To address long-term effects of pesticides on amphibian reproduction, the reproductive capacity of five common toad (*Bufo bufo*) populations in the viticultural landscape of Palatinate in Southwest Germany was investigated along a pesticide gradient (Figure 2). For this, reproductively active adults were captured during their spawning season and kept as pairs in net cages in the study ponds until spawning (n = 5 to 14 per pond). As measures of reproductive capacity, fecundity, fertilization rate, offspring survival until the free-swimming Gosner Stage 25 (GS; Gosner, 1960), and offspring size (tadpole length) at GS25 were investigated. To determine the fecundity, the number of laid eggs per female was counted. Because fecundity is known to increase with female size (Banks and Beebee, 1986; Reading, 1986), the ratio of the amount of laid eggs and the body mass of the females after spawning (eggs/g body mass) was calculated. To estimate the fertilization rate and offspring survival, approximately 90 eggs of each clutch were randomly removed from each egg string and kept in plastic aquariums filled with FETAX medium (Dawson and Bantle, 1987) in a climate chamber under controlled conditions. Developing eggs were counted to calculate the fertilization rate. As soon as all

tadpoles reached GS25, the proportion of embryos that survived to this stage was counted to estimate the offspring survival. Furthermore, the lengths of twelve randomly selected tadpoles of each sample were determined to estimate the offspring sizes.

To compare environmentally relevant concentrations to regulatory acceptable concentrations, acute and chronic RACs were calculated for all detected pesticides based on fish toxicity values from the PPDB (acute: LC50, chronic: NOEC; Agriculture and Environment Research Unit of the University of Hertfordshire, 2013). As uncertainty factors, 100 was used for the acute RAC and 10 for the chronic RAC as recommended for aquatic organisms by EFSA (2013).

4 SUMMARY AND GENERAL DISCUSSION OF FINDINGS

4.1 RQ-1 Aquatic toxicity: Suitability of surrogate organisms for Central European amphibians in acute aquatic toxicity tests

Sensitivity between native species varied by a factor of five for Folpan (Figure 3) and by a factor of eleven for Avaunt (Figure 4). The detected differences may be due to different modes of action and physiological properties of the species. The fungicide folpet acts as cell division inhibitor of many microorganisms with a multi-site activity (Agriculture and Environment Research Unit of the University of Hertfordshire, 2013) whereas the insecticide indoxacarb is a sodium channel blocker that acts *via* contact and stomach action (Agriculture and Environment Research Unit of the University of Hertfordshire, 2013). Interestingly, the most sensitive species to Folpan *Pelophylax* sp. was the least sensitive species to Avaunt. On the other hand, *B. bufo* was the most sensitive species to Avaunt but the least sensitive species to Folpan indicating a complete reversal of these two species in sensitivity. Harris et al. (2000) observed a lower sensitivity of *B. americanus* embryos towards the fungicide mancozeb than *R. pipiens* embryos but reverse results for the insecticide endosulfan. These different results show that no general conclusion can be drawn about more or less sensitive species and that the sensitivity differences cannot be defined only by family or pesticide class.

Xenopus laevis was the second most tolerant species to Folpan with a nearly five times lower sensitivity than the most sensitive amphibian species *Pelophylax* sp. For Avaunt, *X. laevis* was the second most tolerant amphibian species with a nearly nine times lower sensitivity than the most sensitive species *B. bufo*. However, *X. laevis* might be used as surrogate for acute, aquatic toxicity tests and risk assessments of Central European amphibians when applying a minimum uncertainty factor of 9 that covers the higher sensitivities of the tested native species.

Oncorhynchus mykiss showed the highest sensitivity to Folpan (0.256 mg Folpan/L) with a 1.8-fold higher sensitivity than *Pelophylax* sp., thus indicating a suitable surrogate for aquatic stages of Central European amphibian species for this fungicide. The LC50 of 7.0 mg Avaunt/L for *O. mykiss* indicates the second lowest sensitivity with an 8.1-fold lower sensitivity than the most sensitive amphibian species *B. bufo*. HC5 values for the European amphibian species were determined at 0.52 mg Folpan/L and 0.83 mg Avaunt/L. Assuming an aquatic Tier 1 risk assessment, the recommended uncertainty factor of 100 for fish acute toxicity tests (EFSA, 2013) would lead to RACs of 2.56×10^{-3} mg Folpan/L and 0.07 mg Avaunt/L. These RACs would cover the sensitivity of all tested amphibian species. They also cover the determined

HC5 values of the native amphibian species which are 200 and 12 times higher than the determined RACs for fish. Thus, considering the assessment factor of 100, acute toxicity data of standard fish species (here *O. mykiss*) have been found suitable to cover the acute sensitivity of aquatic amphibian stages of the investigated amphibian species in an early hatchling stage for two current-use pesticides.

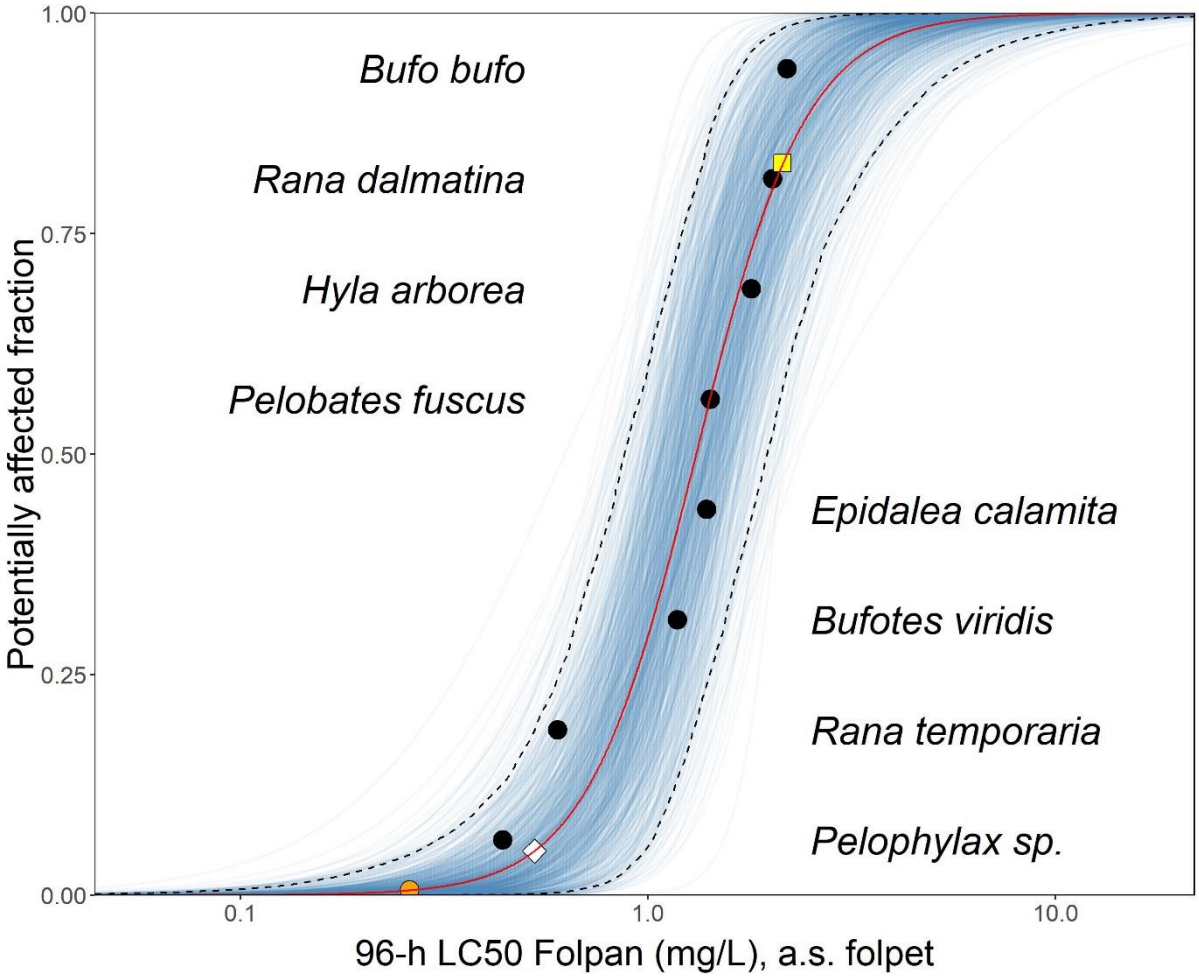

Figure 3. Species sensitivity distribution of Folpan calculated from European amphibian sensitivities (red line). ● denote 96-h LC50 values of German amphibian species. For better comparison, the determined 96-h LC50 values of *Xenopus laevis* (□) and the 96-h LC50 literature value of *Oncorhynchus mykiss* (○) were included. Species names are aligned in ascending order from bottom to top on the same y-axis coordinate as their respective LC50 value. Dashed lines represent parametric bootstrap 95% confidence intervals (1000 iterations) of native amphibian species. Blue lines display all parametric bootstrap samples of native amphibian species. ◇ marks the HC5 value for native amphibian species. **[modified from Appendix A.1]**

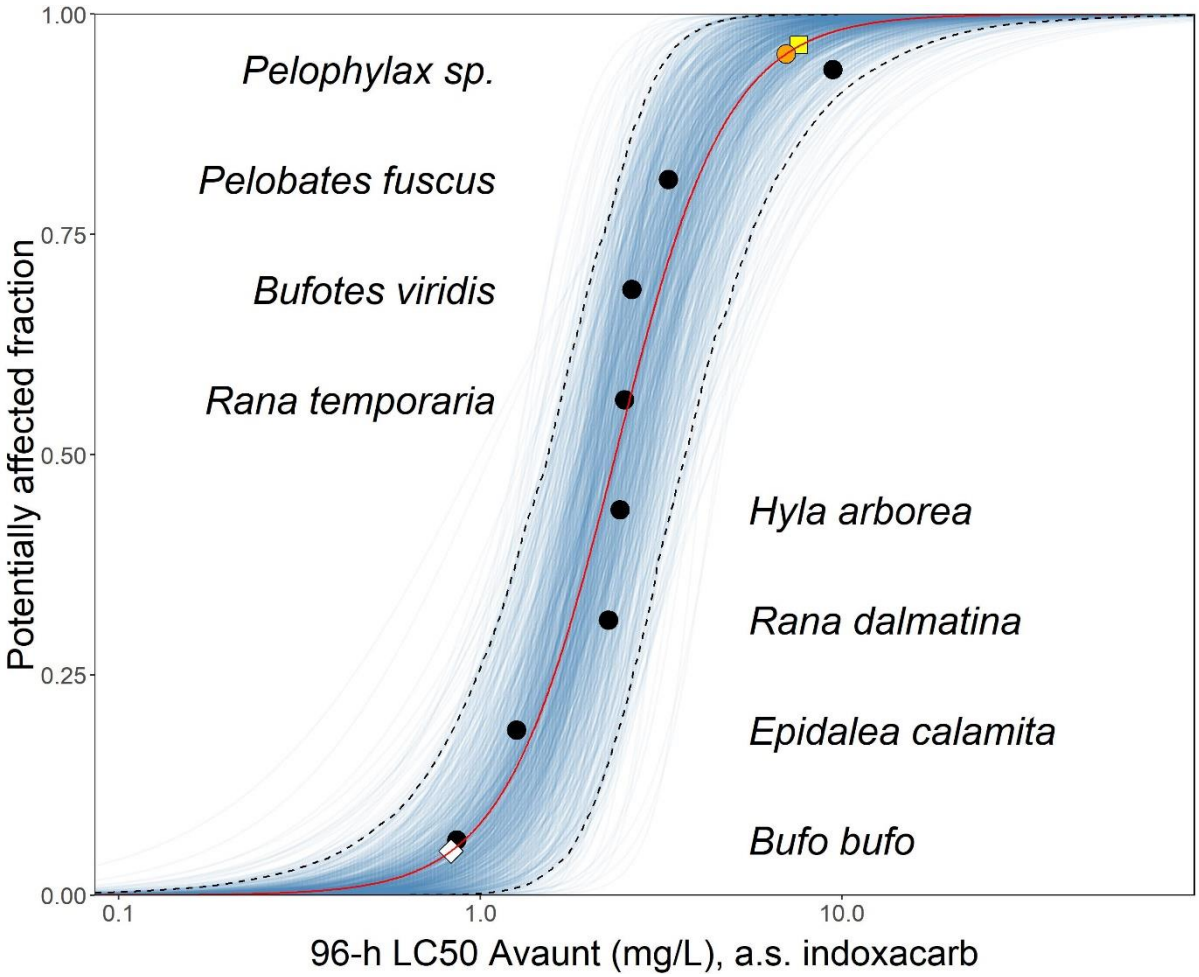

Figure 4. Species sensitivity distribution of Avaunt calculated from European amphibian sensitivities (red line). ● denote 96-h LC50 values of German amphibian species. For better comparison, the determined 96-h LC50 values of *Xenopus laevis* (□) and the 96-h LC50 literature value of *Oncorhynchus mykiss* (○) were included. Species names are aligned in ascending order from bottom to top on the same y-axis coordinate as their respective LC50 value. Dashed lines represent parametric bootstrap 95% confidence intervals (1000 iterations) of native amphibian species. Blue lines display all parametric bootstrap samples of native amphibian species. ◇ marks the HC5 value for native amphibian species. **[modified from Appendix A.1]**

4.2 RQ-2 Formulation toxicity: Aquatic and terrestrial effects of a herbicide formulation and its components

4.2.1 Aquatic toxicity

The determined LC50 values of the active substance cycloxydim, the co-formulants docusate and solvent naphtha, the formulation Focus, its adjuvant Dash and the combination of both are given in Table 3. The active substance cycloxydim was least toxic (LC50 > 100 mg a.s./L) to the *R. temporaria* tadpoles. The two co-formulants led to LC50 values of 62.4 mg docusate/L and 10.2 mg solvent naphtha/L. This indicates that these compounds significantly contribute to the formulation toxicity. Exposure to the formulated product Focus resulted in a LC50 of 29.4 mg Focus/L which is six times higher than the LC50 of the adjuvant Dash (4.56 mg Dash/L). Combination of the formulation Focus with its adjuvant Dash as an equitoxic mixture significantly increased toxicity to *R. temporaria* tadpoles (LC50 of 2.44 mg Focus + Dash/L). Except for Dash, all determined amphibian LC50 values were lower than the fish LC50 values (Table 3). Despite the lower LC50 for Dash, for acute environmental risk assessment purposes, fish LC50 values would cover the determined amphibian sensitivity after application of the recommended uncertainty factor of 100 for acute aquatic toxicity (EFSA, 2013).

To allow predictive mixture toxicity calculations for *R. temporaria*, the LC50 of cycloxydim was set to the highest tested concentration of 100 mg/L with a content of 10.8% in the formulation. Since the safety data sheet of Focus only provides imprecise content ratios of the co-formulants, calculations were conducted using ratios of 50% and 60% for solvent naphtha as well as 2% and 5% for docusate. The different ratios of docusate did not influence the outcome, but solvent naphtha ratio changes did. The predicted LC50 values ranged from 16.5 mg/L (60% solvent naphtha, 2% and 5% docusate) to 19.9 mg/L (50% solvent naphtha, 2% and 5% docusate) which is 32-44% lower than the measured LC50 of Focus (Table 4). The calculated MDR values were 0.56 and 0.68 for the formulation toxicity, thus indicating neither a synergistic nor an antagonistic effect but an additive effect. Thus, the predicted Focus LC50 based on single component LC50 values would cover the aquatic amphibian sensitivity to the formulation. However, due to the significant effect of solvent naphtha content on toxicity, co-formulants exhibiting high toxicities themselves should be considered as a priority in the ERA of pesticide formulations.

Table 3. Measured aquatic 96-h LC50 values of investigated chemicals with 95% confidence intervals and standard error for *Rana temporaria* and literature 96-h LC50 values for fish. Because no mortality of 50% for cycloxydim was achieved, no dose-response model could be fitted to the respective data. The determined LC50 values were significantly different from each other. **[Appendix A.2]**

Substance	LC50 [mg/L]	Lower 95% CI [mg/L]	Upper 95% CI [mg/L]	Standard error [mg/L]	Fish LC50 [mg/L]
Cycloxydim	> 100	n.a.	n.a.	n.a.	> 220 [1]
Docusate	62.37	61.99	62.75	0.19	49 [2]
Solvent naphtha	10.22	9.56	10.88	0.32	2.0 [3]
Focus	29.40	27.58	31.22	0.89	20.4 [1]
Dash	4.56	4.30	4.83	0.13	22 [1]
Focus + Dash	2.44	2.43	2.45	0.01	n.a.

n.a. = not applicable.

[1] Determined for *Oncorhynchus mykiss* (Agriculture and Environment Research Unit of the University of Hertfordshire, 2013; BASF, 2016, 2018).

[2] Determined for *Danio rerio* (Sigma-Aldrich, 2018).

[3] Determined as LL50 (lethal loading rate of water accommodated fractions; water accommodated fractions are media prepared *via* low energy mixing of a poorly soluble test material such as oil; Aurand and Coelho, 2005) for *O. mykiss* (DHC, 2018).

The predicted mixture toxicity of Focus and Dash in an equitoxic mixture led to a predicted LC50 of 7.90 mg/L, which is three times higher than the measured LC50 value, thus underestimating the toxicity of a combined exposure to the formulation and the adjuvant (Table 4). However, the calculated MDR of 3.24 indicates an additive effect and no synergistic effect. The determined underestimation might be due to the additional toxicity of Dash that consists of a surfactant, nonfatty acid methyl ester, and oleic acid (BASF, 2016). Toxicity of mixtures containing surfactants and pesticides has been found to be hardly predictable (Lewis, 1992) as the interaction of surfactants with other chemicals affects different functions and multiple cellular response targets, which generate a complex cascade of events in organisms that cannot be easily summarized (Wei et al., 2009). Therefore, an uncertainty factor should be applied in the ERA of formulations and adjuvants to ensure no unacceptable risk to amphibians.

Table 4. Predicted mixture LC50 values and calculated model deviation ratio (MDR) of *Rana temporaria* tadpoles exposed to a combination of the active substance cycloxydim and the co-formulants solvent naphtha and docusate with different content ratios as well as a combination of Focus and its adjuvant Dash. **[Appendix A.2]**

	SN:D ratio 60:5 and 60:2	SN:D ratio 50:5 and 50:2	Focus:Dash ratio 50:50
Predicted mixture LC50 [mg/L]	16.5	19.9	7.9
Measured mixture LC50 [mg/L]	29.4	29.4	2.44
MDR	0.56	0.68	3.24

SN = solvent naphtha, D = docusate

4.2.2 Terrestrial toxicity

Dermal exposure to soil contaminated with 10% of the recommended FR did not lead to any mortality. After exposure to 100% FR of the substances, no mortality was observed for the control, Focus, Dash and both in combination. Exposure to 5% and 2% of docusate led to 67% and 42% mortality. Solvent naphtha led to 100% mortality after 60% and 40% exposure.

As docusate acts as a surfactant, it decreases the surface tension and thus the barrier of membranes, allowing penetration of water and therefore potentially also of the test solution into the body (Brunton et al., 2018). This membrane modification might have led to docusates GHS classification as “causes serious eye damage” and “causes skin irritation” (Sigma-Aldrich, 2018). The high acute toxicity of solvent naphtha might be caused by its GHS classification as “may be fatal if swallowed and enters airways” (DHC, 2018). On the one hand, inhalation toxicity is especially relevant for amphibians because of their high respiration rate based on their metabolic rate which is increased due to their poikilothermy (Halsey and White, 2010). On the other hand, the entire skin of terrestrial amphibian stages is a respiratory organ and for small individuals with a high surface-to-volume ratio, skin breathing covers an essential part of respiration (up to 30% of O_2 uptake and 70% CO_2 elimination; Burggren and Moallf, 1984). Thus, adverse effects on lung as well as dermal respiration might be the reason for the high toxicity of solvent naphtha. Interestingly, no mortality was observed after exposure to the formulation including docusate and solvent naphtha. This might either indicate an interaction of compounds in the formulation or an alteration of volatility or surfactant properties of the co-formulants in the formulation.

Increased toxicity of solvent naphtha in pesticide formulations to terrestrial amphibian stages was also determined by Brühl et al. (2013). They determined 100% mortality of juvenile *R. temporaria* after direct overspray with a fungicide formulation containing the active substance pyraclostrobin and 67% solvent naphtha. On the contrary, only 20% of the juveniles died after overspray with a pyraclostrobin formulation containing <25% solvent naphtha. These results confirm our findings that solvent naphtha is highly toxic for terrestrial stages of amphibians.

Exposure to soil contaminated with 10% FR of the investigated components led to significantly declined moved distances by juveniles of the cycloxydim and the combined Focus and Dash treatment group in comparison to the control (Figure 5). In this context the non-significant gradual decrease in moved distance by juveniles exposed to Focus, Dash and the combination of Focus and Dash becomes apparent, indicating an enhanced sublethal toxicity by the addition of the adjuvant Dash. Lower activity due to pesticide exposure might play an important role due to the juveniles´ key role in the dispersal of amphibians (Cushman, 2006). Thus, the reduced activity might further contribute to local amphibian declines. In addition, it could lead to an impaired predation behaviour as it was observed in RQ-3 for juvenile *R. temporaria* after exposure to Folpan WDG. Such an effect might lead to a decreased survival that further impairs overall population survival chances.

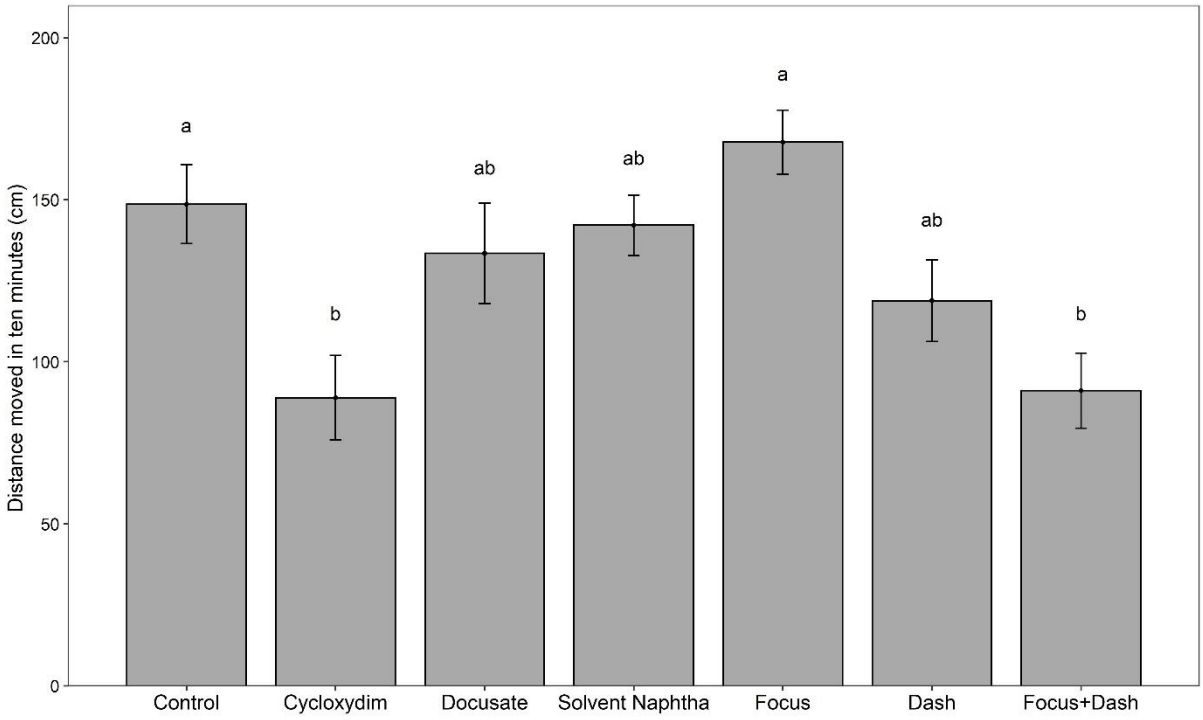

Figure 5. Mean moved distance ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$). **[Appendix A.2]**

Body mass declines were observed after exposure to 10% FR of every test solution except for cycloxydim when compared to the control group (Figure 6). A smaller body mass might represent an increased risk of predation and low survivorship at maturity (Berven and Gill, 1983; Smith, 1987). Declines might have been developed as hydration loss due to irritated or damaged skin caused by the co-formulants as these were present in every test solution except for cycloxydim alone. With respect to the great importance of water for amphibians, this hydration loss might indicate stress regarding the osmoregulation and thus affecting vital functions of the juveniles (Shoemaker and Nagy, 1977). In addition, as solvent naphtha and docusate are included in the pesticide formulations, they might directly affect the amphibian membrane. Therefore, cycloxydim alone might have been excluded from any body burden in the single compound exposure.

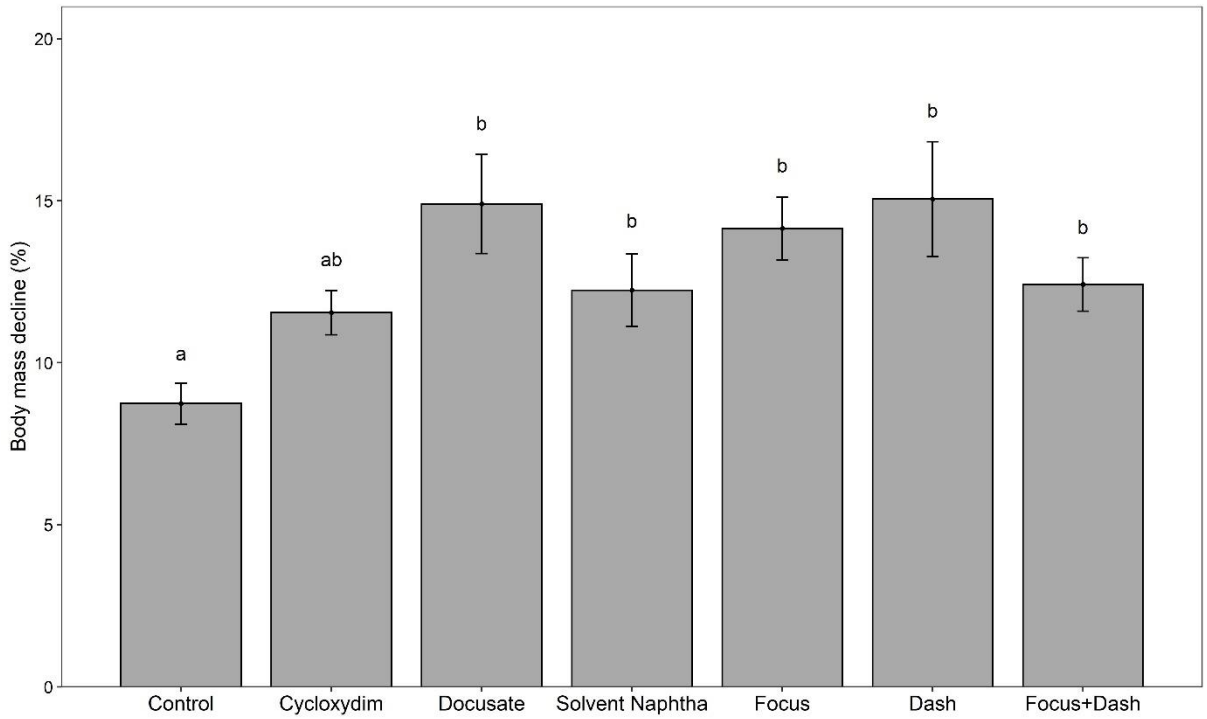

Figure 6. Relative mean body mass decline ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$). **[Appendix A.2]**

4.3 RQ-3 Terrestrial toxicity: Lethal and sublethal effects of terrestrial dermal fungicide exposure

Dermal exposure over 48 hours to both tested fungicide formulations led to mortalities in *R. temporaria* juveniles ranging from 17-100% for Folpan WDG and from 13-17% for Folpan SC (Figure 7). No correlation could be found between the previous aquatic exposure (SumTU)

and mortality after dermal exposure to Folpan WDG. It is remarkable that dermal exposure of soil contaminated with only half of the recommended FR of the most common viticultural fungicide in Germany results in such high mortality levels. Although not statistically significant, detected mortalities of 17-50% (Figure 7) need to be considered as ecologically relevant. Due to these mortality rates, adverse effects on populations seem likely, which might have contributed to existing population declines, especially on a local level. High juvenile mortality rates were also observed following terrestrial exposure to another phthalimide fungicide, captan (Brühl et al., 2013). Brühl et al. (2013) recorded mortalities of 100% of juvenile *R. temporaria* seven days after direct overspray with a captan formulation at 100% of the recommended FR as well as 40% mortality at already 10% FR which shows that phthalimide fungicides might be very harmful to terrestrial amphibian stages.

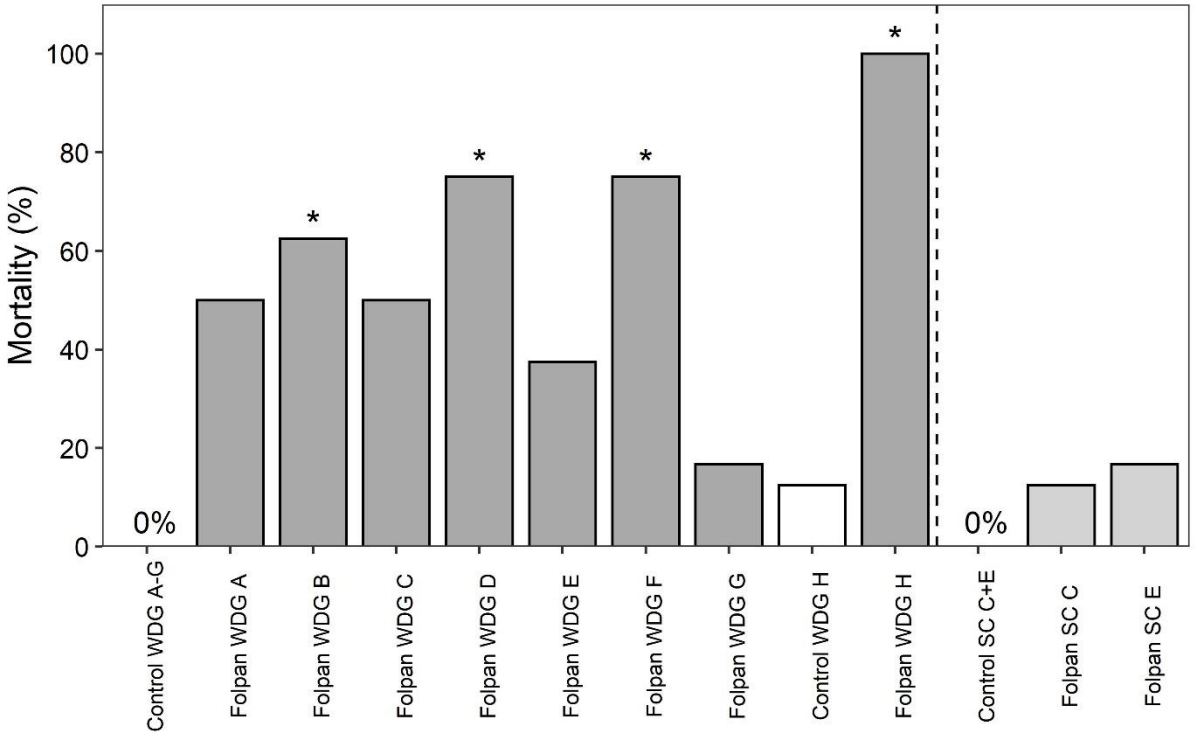

Figure 7. Mortality of juvenile *Rana temporaria* of each pond after 48-h exposure to Folpan WDG (dark-gray) and SC (light-gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. Asterisks (*) denote statistically significant difference ($p < 0.05$) between control and respective treatment. **[Appendix A.3]**

Exposure to either of both formulations did not induce statistically significant body mass declines (Figure 8). No effect of the rearing ponds on the mass decline was determined.

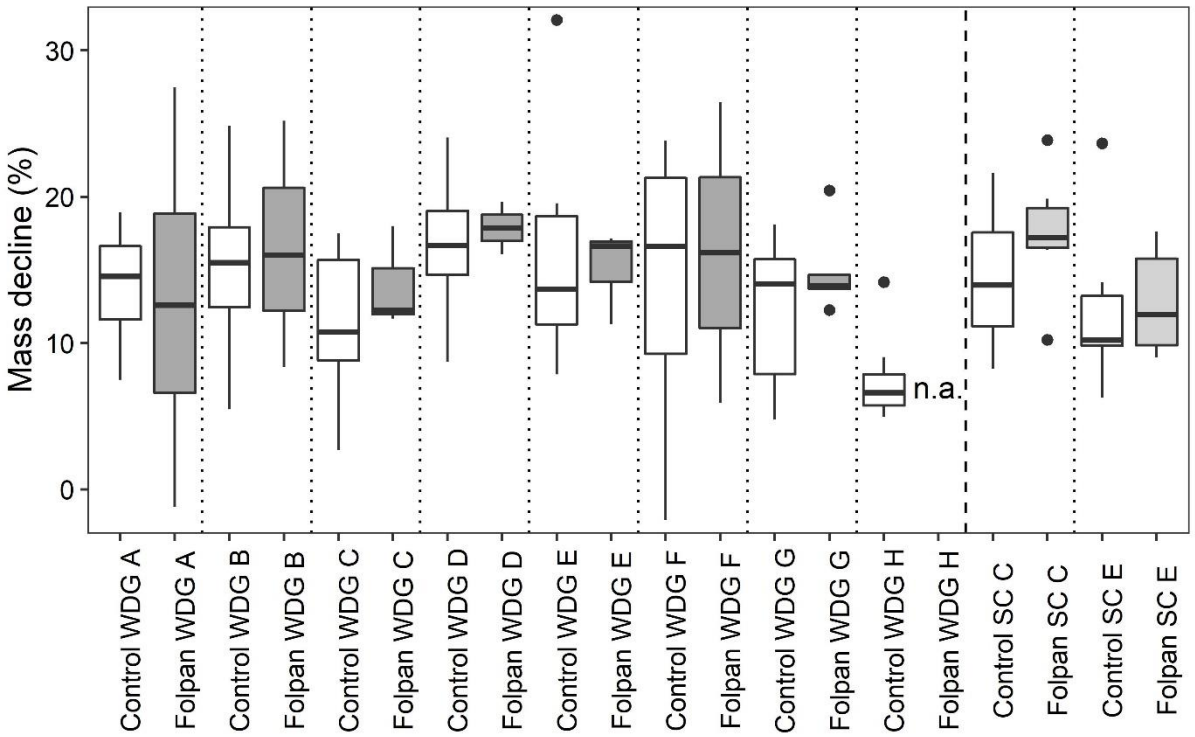

Figure 8. Relative mass decline of juvenile *Rana temporaria* of each pond after 48-h exposure to Folpan WDG (dark-gray) and SC (light-gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. No statistically significant difference ($p < 0.05$) between control and respective treatment were found. n.a. = not applicable. **[Appendix A.3]**

Folpan WDG induced median decreased locomotor activity ranging from 9-73% (Figure 9). Significantly decreased activity between the treatment and control group was determined only for pond G. Exposure to Folpan SC resulted in a non-significant 85% reduction and a 32% increase of the median total distance moved for pond C and E, respectively. No effect of the rearing ponds on the distance moved after exposure to Folpan WDG and no correlation for the influence of SumTU on the distance moved after exposure to Folpan WDG was determined. Although not always statistically significant, the determined reduced activity could further contribute to local amphibian declines due to the reasons illustrated in RQ-2.

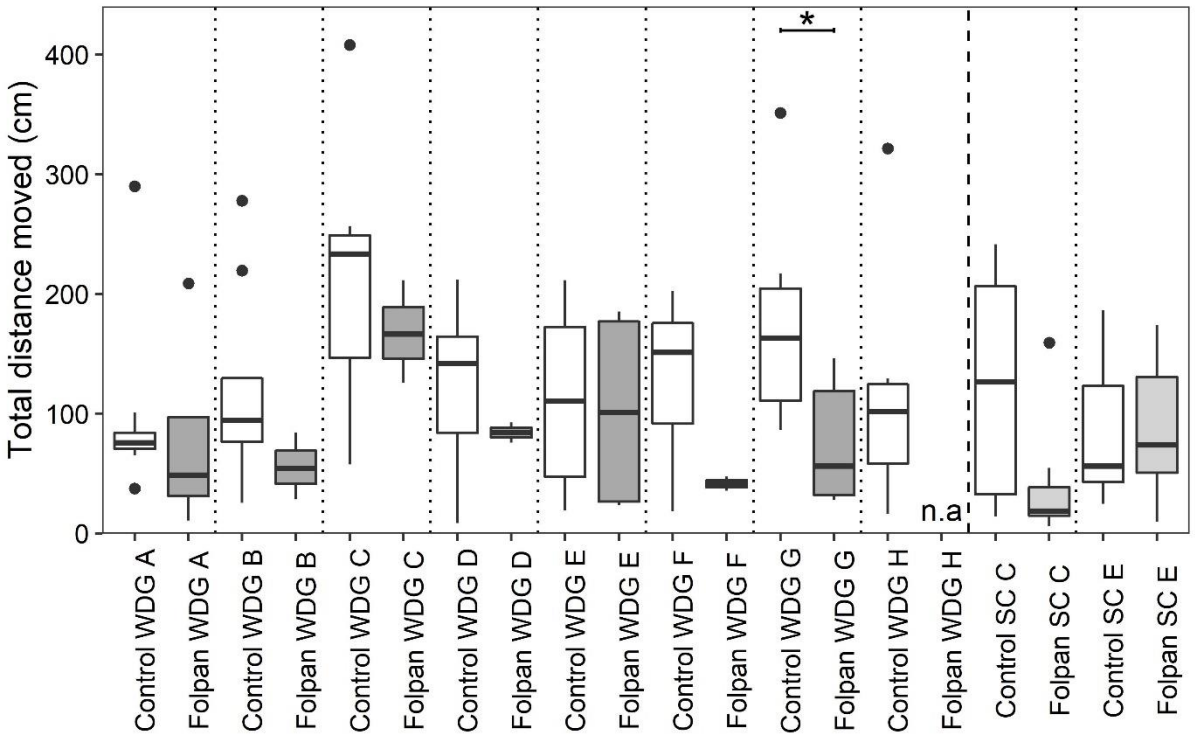

Figure 9. Total distance moved of juvenile *Rana temporaria* of each pond after 48-h exposure to Folpan WDG (dark-gray) and SC (light-gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. Asterisks (*) denote statistically significant difference ($p < 0.05$) between control and respective treatment. **[Appendix A.3]**

The feeding behaviour test revealed a non-significant mean reduction of 20% of consumed *D. melanogaster* after exposure to Folpan WDG and a significant mean reduction of 47% after exposure to Folpan SC (Figure 10). Furthermore, for both fungicides non-significant mean increases of 50% and 169% in necessary time to catch the first *D. melanogaster* were observed (Figure 10). These results may even underestimate this effect because only a limited number of flies to prey on were offered, and the control group might have eaten more flies than offered. Different results of Cusaac et al. (2017) and Webber et al. (2010) who did not find any effect on feeding behaviour after exposure to carbaryl and pyraclostrobin may be due to different species sensitivities (Bridges and Semlitsch, 2000), different modes of action of the pesticides, different spray scenarios, and use of various underground substrates like wet towels or soil compositions. This highlights the need for standardized protocols for post-metamorphic, terrestrial amphibian testing to allow a more precise comparison among studies and pesticides.

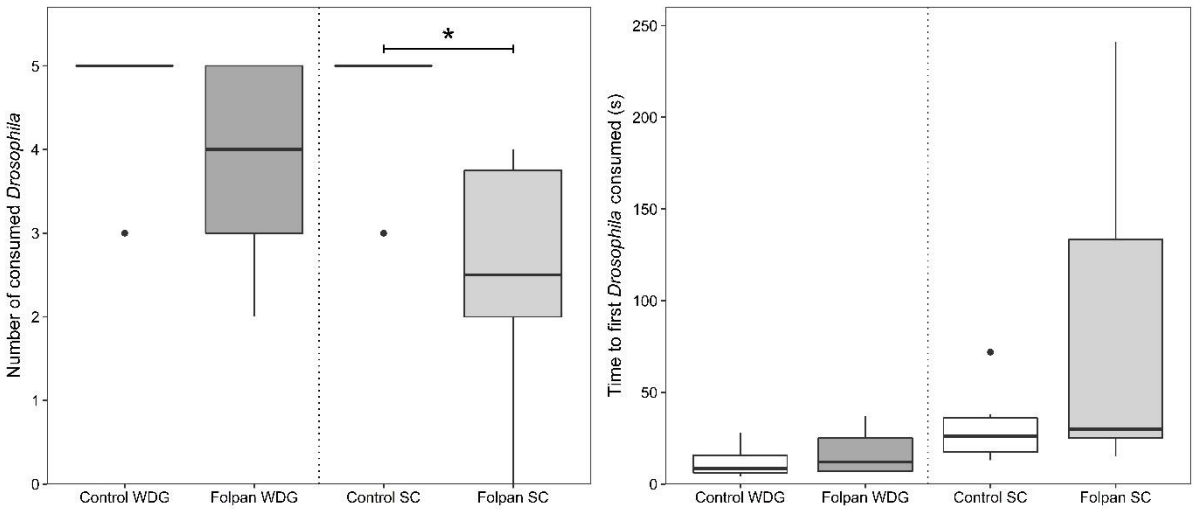

Figure 10. Number of consumed *Drosophila melanogaster* of juvenile *Rana temporaria* (left) and time that juveniles needed to catch the first *D. melanogaster* (right) in control and treatment group of pond E after 48-h exposure to Folpan WDG (dark-gray) and Folpan SC (light-gray). Asterisks (*) denote statistically significant difference ($p < 0.05$) between control and treatment group. **[Appendix A.3]**

In both folpet tests, the same amount of active substance was applied to the soil. Thus, the faster and significantly higher mortality after exposure to Folpan WDG compared to Folpan SC indicates that the formulation-specific toxicity must be due to differing co-formulants as it was also observed in RQ-2. Folpan SC was expected to be more toxic because of the properties of its additives, which are classified as eye-irritating and skin-sensitizing in contrast to the additive of Folpan WDG, which is classified as chronically toxic to aquatic organisms. Considering the aquatic toxicity of both formulations to *Daphnia magna*, Folpan WDG has a 5-times lower 48-h LC50 for *Daphnia magna* than Folpan SC (0.68 vs 3.9 mg/L, respectively; Adama, 2015, 2016) indicating that Folpan WDG may be more toxic than Folpan SC nonetheless. However, the varying toxicity may also be induced because of the additives altering the absorption and metabolism properties of the formulation and not because of their own toxicity. Therefore, no general conclusion on the actual reason for this difference in toxicity can be drawn in this thesis. Investigating actual concentrations of co-formulants might help to draw conclusions about the reasons in future research.

Due to the higher content of folpet in the granule formulation (78-85% a.s.) than in the suspension concentration (38-42% a.s.) and the high detected mortality of Folpan WDG in the terrestrial toxicity tests, the risk assessment calculation was performed for Folpan WDG in vine. Eight different scenarios including the lowest and highest application rate proposed by the

manufacturer (0.3 and 1.6 kg/ha; Adama, 2021) as well as in-field, off-crop, interception and off-crop drift scenarios were calculated according to the calculation steps described in chapter 3.4.

According to the EFSA peer review conclusion of folpet (EFSA, 2009a), the BCF was determined to be 56 L/kg in bluegill fish (*Lepomis macrochirus*). As no LC50 for *L. macrochirus* was determined, the LC50 of folpet for *O. mykiss* (0.233 mg/L; EFSA, 2009a) was used for the calculations leading to a $LD50_{fish}$ of 12.3 mg/kg and a corresponding $LD50_{amphibian}$ of 14.3 mg/kg. The calculated TERs ranged from 0.4-7.7 for in-field scenarios and from 16-276 for off-crop scenarios (Table 5). The TERs of the in-field scenarios considering both, the highest and lowest application rate, were lower than the trigger value of five. Furthermore, the TER of the in-field scenario of the highest application rate with a crop interception of 70% was lower than five.

Table 5. Risk assessment calculations for juvenile terrestrial amphibian stages dermally exposed to folpet based on spray application data of Folpan WDG. [unpublished data]

Scenario	Application rate [kg/ha]	Dose (full overspray) [mg/kg]	Exposure without interception [%]	Crop interception [%]	Dose refined [mg/kg]	TER
In-field	1.6	34.9	100	0	34.9	**0.4**
In-field	0.3	6.54	100	0	6.54	**2.3**
In-field	1.6	34.9	100	70 [2]	10.5	**1.4**
In-field	0.3	6.54	100	70 [2]	1.96	7.7
Off-crop	1.6	n.a.	2.77 [1]	0	0.97	16
Off-crop	0.3	n.a.	2.77 [1]	0	0.18	83
Off-crop	1.6	n.a.	2.77 [1]	70 [2]	0.29	52
Off-crop	0.3	n.a.	2.77 [1]	70 [2]	0.05	276

TER = toxicity exposure ratio; TERs lower than the trigger value of five are highlighted bold.
n.a. = Not applicable.

[1] Off-crop drift during single spray application according to FOCUS (2001).

[2] Crop interception according to EFSA (2009b) in vine in BBCH stage 61 and higher (proposed application stage according to manufacturer; Adama, 2021).

These results show that the high toxicity of folpet to terrestrial amphibian stages could have been predicted using the available calculation tool by Weltje et al. (2017). As observed in this study, exposure to only 50% FR of folpet can lead to lethal and sublethal effects. Even an in-field crop interception of 70% would not be safe for juvenile amphibians. To avoid severe adverse effects on amphibian populations, pesticides that indicate such high acute toxicity using the calculation tool should be only used when there is no spatial and temporal overlap of pesticide application and amphibian migration. Amphibians have not been found to avoid soil contaminated with Folpan WDG (Leeb et al., 2020b). Therefore, it cannot be assumed that amphibians would rather use off-crop migration corridors that seem to have an acceptable risk as the TERs are higher than the trigger value of five.

4.4 RQ-4 Reproduction toxicity: Effects of long-term pesticide exposure on reproduction

Reproductive capacity of *B. bufo* populations was affected by pesticide contamination of the studied ponds (Figure 11 A-D).

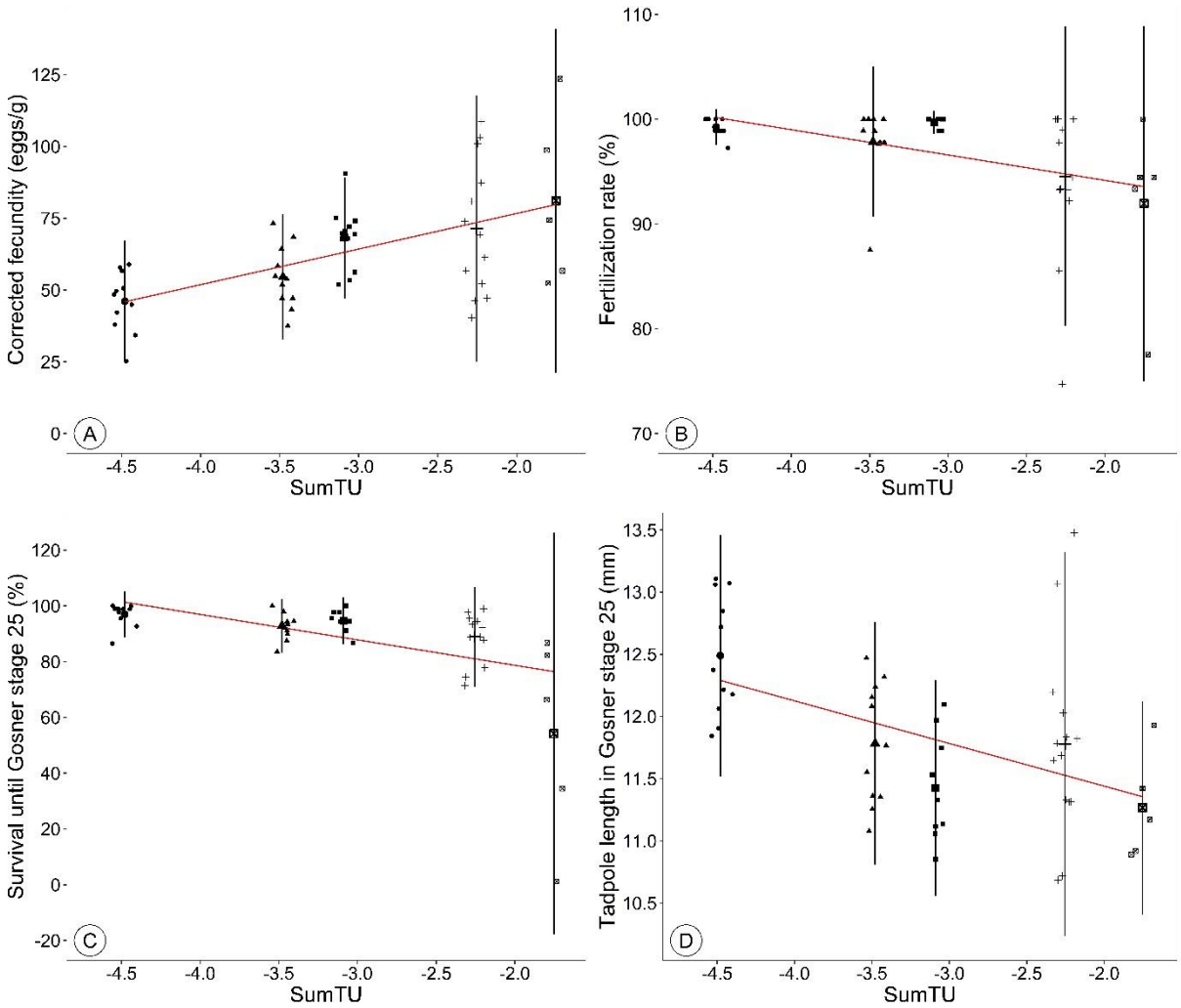

Figure 11. Dependence of fecundity (A), fertilization rate (B), offspring survival until Gosner stage 25 (C) and offspring size in Gosner stage 25 (D) on pesticide contamination of breeding ponds (maximum sum of toxic units, SumTU). Fecundity was corrected for the body mass of females after spawning (eggs/g body mass). For each pond, means and standard deviations are presented. **[modified from Appendix A.4]**

Mean fecundity ranged from 49 to 74 eggs/g body mass and showed a positive correlation with increasing SumTU (Figure 11 A). Toads of the highest contaminated pond E showed on average a 1.5 times higher fecundity than toads of the uncontaminated pond A. In comparison to the present study, Bókony et al. (2018) did not observe any effect on the fecundity of common toads in agricultural ponds compared to natural ponds. Because the female body mass correlated with the number of eggs and both of these correlated with SumTU, the increased fecundity may be based on the higher female body masses in the contaminated ponds. The increased body sizes might either suggest a potential adjustment during aging or some habitat specificities in the agricultural landscape may enhance body size. For example, smaller population densities in

agricultural landscapes might decrease intra- and/or interspecific competition leading to larger individuals (Bishop et al., 1999; Guillot et al., 2016; Janin et al., 2011).

Fertilization rate showed a mean decrease of 4.5% and a positive correlation with increasing SumTU (Figure 11 B) while the rate was not affected by the parental body masses or the number of laid eggs. There are several reasons that may have led to the observed decreased fertilization rate. Due to the increased number of eggs per female, the male fertilization success may have been reduced. But also behavioural impairments during mating could lead to decreased fertilization rates. Hayes et al. (2010) observed a reduced success of amplexus in male *X. laevis* exposed to the herbicide atrazine and thus a lower proportion of fertilized eggs for atrazine exposed males. Also endocrine disruptive properties of pesticides may have led to this decrease for example due to impaired spermatogenesis (Hayes et al., 2010) or sexual differentiation of testes (Tavera-Mendoza et al., 2002b). Another reason may be an effect on female sexual development such as altered ovarian steroidogenesis, reduced progesterone production (Orton et al., 2009), inhibition of oviposition, and maturation of oocytes (Pickford and Morris, 2003; Tavera-Mendoza et al., 2002a)

Offspring survival and length in GS25 negatively correlated with increasing SumTU and revealed mean decreases of 32.6% and 10.7% (Figure 11 C and 11 D). Furthermore, offspring size was negatively correlated with the number of laid eggs per female. The reduced tadpole lengths could lead to further impairments since body size is a critical determinant of individual fitness (Wells, 2007). A smaller tadpole size can lead to reduced size at metamorphosis and thus to a decreased survivorship of the first hibernation (Üveges et al., 2016) and until maturity as well as delayed achievement of reproductive size (Smith, 1987). Reduced body size is also a disadvantage for adults with regard to their reproduction because it affects female fecundity and male mating success (Banks and Beebee, 1986; Davies and Halliday, 1979; Reading et al., 1991).

On the one hand, reduced offspring size may be a long-term consequence of chronic pesticide pollution over several generations. Transgenerational effects were observed in rats after the exposure to endocrine disruptors by Anway et al. (2005) which detected a decreased spermatogenic capacity in cell number and viability as well as an increase of male infertility in four tested generations. Thus, early-life exposure of parents might have led to impaired offspring. To verify the proposed reasons of reproduction impairments regarding endocrine disruptive effects, tissue analyses of e. g. thyroids and gonads should be investigated in future research.

On the other hand, the reduced offspring size originating from highly contaminated ponds may be a cost of an evolutionary adaptive resistance (Whitehead et al., 2012) or of detoxification processes of contaminants (Rix et al., 2016) as it was observed for fish populations which evolved tolerances to toxic pollutants (Meyer and Di Giulio, 2003; Whitehead et al., 2012). Their offspring showed reduced growth rates and were more susceptible to other stressors compared to offspring from a non-contaminated site (Meyer and Di Giulio, 2003). Similar trade-offs may be responsible for the smaller tadpoles of the more contaminated ponds. Adult toads of these ponds may invest more resources into the production of egg jelly coat material to provide a better protection against pesticides. These resources may have in turn not be invested into larger ova (Podolsky, 2004) which may have led to smaller tadpoles such as determined by Kaplan (1980). The higher egg production in contaminated ponds may be discussed as an adaptation to increase fitness by counterbalancing negative pesticide effects on embryo and tadpole development by an increased number of eggs.

Comparison of detected pesticide concentrations to RACs revealed a conspicuous toxicity of exposure to the fungicides folpet and famoxadone in the highest contaminated pond E. The chronic RAC of folpet was 0.81 µg/L which is 5.6 times lower than the highest detected folpet concentration (4.53 µg/L). The chronic RAC of famoxadone was 0.14 µg/L which is 1.1 times lower than the highest detected famoxadone concentration (0.15 µg/L). The acute RAC of 0.11 µg/L for famoxadone was 1.4 times lower than the highest detected concentration. Not only the revealed risk of a chronic toxicity for aquatic vertebrates in pond E exposed to folpet and famoxadone might lead to adverse effects but also mixture effects in ponds with up to 19 detected pesticides may contribute to higher toxicities (Relyea, 2009). In addition, it cannot be excluded that even higher concentrations and further pesticides were present in the ponds due to the limited number of water samplings (n = 5) and analysed pesticides (n = 58). Since only one rain event sampling was performed in the present study, peak pesticide concentrations may be underestimated (Neumann et al., 2003). Especially folpet and famoxadone may be present at higher concentrations than detected because they have very short dissipation times in water (DT50 folpet = 0.02 d, DT50 famoxadone = 0.1 d, Agriculture and Environment Research Unit of the University of Hertfordshire, 2013).

5 CONSEQUENCES FOR THE EUROPEAN PESTICIDE RISK ASSESSMENT

5.1 Consequences for aquatic toxicity

The results of RQ-1 and RQ-2 allow to draw conclusions for an acute pesticide risk assessment for aquatic stages of Central European amphibians. Both *X. laevis* and *O. mykiss* seem to be potential surrogate species for the tested amphibian species after application of an appropriate assessment factor. As fish acute toxicity tests need to be performed for registration purposes of pesticides, no further vertebrate testing seems to be necessary. This conclusion accords to the findings of Birge et al. (1985), Fryday and Thompson (2012), and Weltje et al. (2013) that acute toxicity data of standard fish species are suitable to cover the acute sensitivity of aquatic amphibian stages. Whether the chronic aquatic toxicity can be covered by the ERA of fish cannot be answered with the present studies. Weltje et al. (2013) investigated chronic amphibian and fish data for 52 chemicals to which mostly fish were more sensitive than amphibians. However, substances which interfere with metamorphosis specific pathways such as the thyroid hormone activity cannot be identified in fish tests. Next to direct effects on metamorphosis pathways, pesticides have been shown to affect for example time of metamorphosis and body mass at metamorphosis (e.g. Boone, 2008; Gaietto et al., 2014), which might lead to reduced terrestrial fitness and survival as discussed in 4.2 and 4.3. Therefore, it cannot be guaranteed that chronically toxic substances which harm amphibians will be identified in chronic fish toxicity tests.

One possibility to address chronic aquatic effects also on metamorphosis might be an extended amphibian metamorphosis assay (AMA; OECD, 2009), a screening assay intended to empirically identify substances which may interfere with the normal functioning of the hypothalamic-pituitary-thyroid axis in *X. laevis*. At test initiation, tadpoles must be at Nieuwkoop and Faber stage 51 (Nieuwkoop and Faber, 1994), in which already hind limbs emerge. The test is terminated after 21 days neglecting the developmental stage of tadpoles. Next to mortality, the developmental stage, hind limb and snout-vent length, wet body weight and thyroid gland histology are investigated endpoints. This assay could be used as starting point for amphibian metamorphosis investigations as it could be performed as an extended AMA starting with embryonic individuals and terminating after all individuals reached metamorphosis. With such an extension, chronic effects and effects on metamorphosis pathways might be identified reliably.

In RQ-2 it was shown that the aquatic formulation toxicity to amphibians could be also covered by the acute ERA of fish. In addition, the concentration addition model is a helpful tool to predict formulation toxicities not only for fish but also for aquatic amphibian stages. However, due to complex interactions of pesticides and surfactants (Lewis, 1992; Wei et al., 2009) the model cannot be used to predict mixture toxicities in a reliable way as shown for the combination of Focus and Dash. Surfactant adjuvants therefore need special consideration in the future ERA for amphibians, not only in aquatic but especially in terrestrial stages as it will be discussed in 5.2.

It needs to be noted that all aquatic tests performed for this thesis were carried out under controlled laboratory conditions at temperatures of ~21°C. Temperature and other environmental conditions are co-stressors which might change the amphibian sensitivity (Baier et al., 2016; Leeb et al., 2021; Mikó et al., 2015). The fish acute toxicity test with *O. mykiss* is usually performed at temperatures between 10°C and 14°C which is more realistic than temperatures around ~21°C because tadpoles are usually exposed to pesticides in spring and early summer. As the AMA is usually performed at ~22°C a potential influence of temperature needs to be considered in future ERA.

5.2 Consequences for terrestrial toxicity

In general, most studies investigating ecotoxicological responses of amphibians to pesticides focus on larval amphibian stages (Sievers et al., 2019) emphasizing the underrepresentation of terrestrial amphibian stages in ecotoxicological studies (Brühl et al., 2011). The results of RQ-2 and RQ-3 show that there might be lethal and sublethal effects on postmetamorphic amphibian stages after dermal exposure to pesticide formulations. Especially due to the unique skin characteristics of amphibians, these effects cannot be covered by the risk assessment of other terrestrial vertebrates such as birds and mammals. The dermal pathway for birds is not specifically addressed in the terrestrial vertebrate ERA. For mammals, endpoints for dermal toxicity could be derived from tests that are performed in the human toxicological risk assessment for example for worker. However, skins employed in these tests differ clearly from the characteristics of amphibian skin as they include a stratum corneum that is substantially thicker than the stratum corneum of amphibians.

As only a few dermal toxicity studies for terrestrial amphibian stages are available, a general usability for the suggested screening tool of Weltje et al. (2017) cannot be guaranteed. However, and although not addressing sublethal effects, the tool could be used as a basis for

dermal toxicity risk assessment for amphibians. As shown in RQ-2 and RQ-3 the tool needs to be refined with information about potential skin or inhalation toxicity due to the presence of co-formulants such as surfactants in pesticide formulations. *In vitro* data generated for human health assessment (e.g. GHS classification) could provide additional information on dermal toxicity as there might be a relation between skin corrosion and irritation in humans and amphibians *in vitro* (Kaufmann and Dohmen, 2016). Next to these toxicity data, hydrophilic property data such as the octanol-water partition coefficient (log K_{OW}) might be useful non-testing predictors for dermal toxicity as the uptake of pollutants through amphibian skin has been shown to depend on this coefficient (Quaranta et al., 2009). Besides, the soil partition coefficient (K_{OC}) could be included in future assessments as this coefficient has been shown to be an even better predictor to pesticide body burden, bioconcentration, and skin permeability than the octanol-water partition coefficient (van Meter et al., 2014). To allow predictions about the bioconcentration of pesticides is especially important as data about bioconcentration (i.e. BCF) is also needed in the original screening tool of Weltje et al. (2017). However, a BCF is not always available for all pesticides as the bioconcentration of a substance only needs to be assessed for regulatory purposes when the log K_{OW} of a substance is greater than three and it is not rapidly degraded in water (EFSA, 2015). To refine the non-testing approach of Weltje et al. (2017), skin and inhalation toxicity data should be combined with dermal absorption data (Weltje et al., 2018). For this, not only further data but also a validation is needed in future analyses.

Further, it needs to be considered that the poikilothermy of amphibians plays an important role in defining potential toxicity of exposure to pesticides as the metabolic rate, oxygen consumption, and energetic expenditure are directly associated with the environmental temperature. Increased metabolic rates can lead to increased energetic demands and respiratory rates (Halsey and White, 2010) and therefore to an increment of the oral or inhalation uptake (Avery, 1971). Furthermore, increased metabolic rates can lead to higher locomotor activity and thus the risk for pesticide uptake grows. On the one hand, higher metabolic rates also result in more readily metabolised pesticides, which in turn reduces the risk of suffering toxic effects. On the other hand, detoxification has an associated energy cost that can affect other energetic investments, thus comprising other biological functions such as growth, immunity or reproduction. As these detoxification processes are highly affected by metabolic processes, poikilothermy constitutes a key issue, making amphibian toxicokinetics and toxicodynamics different from those of birds and mammals. Therefore, the uptake, metabolism and elimination of pesticides in amphibians need further consideration for a protective ERA.

Although there is a spatial-temporal overlap of terrestrial amphibian stages and pesticide applications (Leeb et al., 2020a; Lenhardt et al., 2015), relevant refinement measures could be applied to the risk assessment. Next to interception by crops for example according to the EFSA guidance for the risk assessment for birds and mammals (EFSA, 2009b), an appropriate focal species selection with corresponding morphology as well as feeding and behavioural data such as the time that amphibians are present in-field or avoidance behaviour (Leeb et al., 2020b) should be considered in future risk assessment approaches.

5.3 Consequences for reproduction toxicity

Although the findings of RQ-1, RQ-2, and RQ-3 show that the acute pesticide toxicity to aquatic and terrestrial amphibians might be either covered by existing ERA approaches or roughly predictable by available tools, further vertebrate studies seem to be inevitable regarding long-term effects on reproduction of amphibians. The findings of RQ-4 support the suggestion of inhibitory effects of current-use pesticides on the reproductive capacity of amphibians, potentially contributing to population declines. Thus, not only acute effects need be addressed in a future ERA for amphibians but also sublethal, chronic effects on reproduction on a population level. Since data involving field scenarios analysing the effects of multiple pesticides on amphibian reproduction are considerably rare, the results of RQ-4 are of significant importance for amphibian conservation in agricultural landscapes.

Currently, no standard test covers the full life-cycle of amphibians and the amphibian specific reproduction biology. Both ecological studies in complex field systems and controlled laboratory experiments are needed to understand underlying mechanisms and modes of toxicity. To analyse reproduction responses and to understand consequences of multigenerational effects of pesticides, standardized methods and endpoints need to be established for amphibians as they already exist for birds and mammals (e.g. OECD, 1984, 2001). When focusing on reproduction neglecting a biphasic life-cycle, the aquatic species *X. laevis* might be a potential model species as it is becomes sexually mature within less than one year (Gasche, 1943; McCoid and Fritts, 1989), whereas Central European anuran species mostly become sexually mature only after several years (Günther, 2009). Another potential model species could be *X. tropicalis* as this species was used in one of the very few multigenerational studies showing transgenerational toxicity of pesticides in amphibians (Karlsson et al., 2021). Relevant endpoints might include adult and progeny weight, fecundity, fertilization rate as well as survival, size, and development of offspring. These tests could be used to compare findings for amphibians to birds and

mammals allowing a conclusion whether further amphibian tests need to be performed for a protective amphibian risk assessment or whether amphibian reproduction can be covered by existing bird and mammal data or individual aquatic and terrestrial amphibian data.

Such investigations need to be set into context with environmentally relevant concentrations. Currently, monitoring data in small, standing water bodies which are used for amphibian reproduction are scarce (Aldrich et al., 2016) as these water bodies are not routinely monitored under the Water Framework Directive. The “Kleingewässermonitoring” (monitoring of small water bodies) executed according to the National Action Plan on sustainable use of pesticides in Germany monitored such small water bodies at 140 field sites in 2018 and 2019. These data could be helpful to assess the actual exposure of amphibians to pesticides in small standing ponds.

6 CONCLUSION AND OUTLOOK

The present thesis provides information on pesticide effects on amphibians and conclusions for a future risk assessment of pesticides. It was clearly demonstrated that the unique characteristics of amphibians make them highly susceptible to pesticides. Extensive evidence was provided that environmentally relevant pesticide exposure negatively affects survival, body mass, behaviour and reproduction of amphibians [RQ-2 – RQ-4, Appendix A.2-A.4]. The combined, chronic aquatic and terrestrial exposure severely affects amphibian populations and therefore contribute to the ongoing amphibian decline. Ecological consequences of amphibian population declines are of particular concern because amphibians are essential members of an ecosystem (Günther, 2009). Due to their high biomass, amphibians are important components of trophic nets, both as consumers of large amounts of food and as prey for top predators, thus representing key elements in the transfer of energy and pesticides not only across trophic boundaries but also across water-land boundaries. Therefore, there is an urgent need for a protective ERA for amphibians exposed to pesticides.

In this thesis it was shown that existing risk assessment approaches can be used as a basis for a future risk assessment for aquatic and terrestrial amphibian stages [RQ-1 and RQ-3, Appendix A.1 and A.3]. However, this thesis only represents a selection of species, developmental stages, pesticides and exposure pathways. Therefore, there are many open research questions and data gaps that need to be addressed to facilitate a protective ERA for amphibians.

Furthermore, this thesis focussed on direct effects of pesticides on amphibians. Besides, indirect effects due to loss of food animals are very likely. Fungicides can change nutritious quality of leaf litter which is used as food source by tadpoles. These changes can have adverse effects on the tadpole development indirectly through bottom-up effects (Bundschuh et al., 2021). As the main prey for terrestrial amphibians is moving arthropods, insecticide applications drastically decrease the food availability to amphibians. Additionally, herbicide applications reduce weed biomass resulting in smaller forage availability to herbivore arthropods which in turn decrease food availability to amphibians. Therefore, to preserve amphibian populations, it is necessary to consider direct as well as indirect effects originating from reduced food sources in a future ERA of pesticides.

Aside from regulatory measures, management measures could help to preserve and promote stable amphibian populations. In Europe, agricultural landscapes with frequent pesticide applications are the dominant type of land in many regions. Although amphibians use farmed

land as temporary habitat, they have also been found to try to avoid these habitats (Leeb et al., 2020a; Miaud and Sanuy, 2005; Salazar et al., 2016). Such an avoidance behaviour might contribute to a fragmentation of used habitats which can lead to reduced gene flow between amphibian populations (Lenhardt et al., 2013; Lenhardt et al., 2017) and therefore to reduced fitness and decreased long-term survival of a population . Therefore, a heterogeneous landscape with buffer strips around ponds as well as uncultivated patches and migration corridors between populations and different habitat types are needed to facilitate avoidance behaviour by amphibians without leading to a habitat fragmentation (Costanzi et al., 2018; Leeb et al., 2020b; Leeb et al., 2020a). Moreover, a less intensive agriculture using biological pest control for example by beneficial insects but also by amphibians would greatly benefit amphibian populations. Given the multiple reasons affecting amphibian populations next to conventional agricultural techniques such as habitat loss and fragmentation, invasive species, diseases, and climate change, maintaining a protective and sustainable environment for amphibians will be a complex challenge in future.

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APPENDICES

Appendix A.1 – Scientific publication 1

Interspecific sensitivity of European amphibians towards two pesticides and comparison to standard test species

Elena Adams, Christoph Leeb, Alexis P. Roodt, Carsten A. Brühl

Abstract

Background: Although debates about the assessment of potential effects of pesticides on amphibians are ongoing, amphibians are not yet considered in the current EU environmental risk assessment of pesticides. Instead, the risk assessment of potential effects on aquatic amphibian life stages relies on use of data of surrogate species like the standard temperate fish species rainbow trout (*Oncorhynchus mykiss*). This assumption is mainly based on the comparison to amphibian species not native to Europe such as the aquatic African clawed frog (*Xenopus laevis*). It remains unclear whether these surrogate species cover semi-aquatic Central European amphibian sensitivities. Therefore, we assessed the acute sensitivity of aquatic stages of eight European amphibian species native in Germany (*Bufo bufo*, *Bufotes viridis*, *Epidalea calamita*, *Hyla arborea*, *Pelobates fuscus*, *Pelophylax* sp., *Rana dalmatina*, *R. temporaria*) towards commercial formulations of the fungicide folpet (Folpan® 500 SC, Adama) and the insecticide indoxacarb (Avaunt® EC, Cheminova). The determined acute sensitivities (median lethal concentration, LC50) were included in species sensitivity distributions and compared to experimentally determined LC50 values of *X. laevis* and literature values of *O. mykiss*.

Results: The results showed that native amphibian sensitivities differed between the tested pesticides with a factor of 5 and 11. Depending on the pesticide, *X. laevis* was five and nine times more tolerant than the most sensitive native amphibian species. Comparing literature values of *O. mykiss* to the experimentally determined sensitivities of the native amphibian species showed that the *O. mykiss* sensitivity was in the same range as for the tested amphibians for the formulation Folpan® 500 SC. The comparison of sensitivities towards the formulation Avaunt® EC showed an eight times lower sensitivity of *O. mykiss* than the most sensitive amphibian species.

Conclusions: A risk assessment using the 96-h LC50 values for fish covers the risk for the assessed aquatic stages of European amphibians after the application of the recommended uncertainty factor of 100 and thus may be adequate for lower tier risk assessment of the studied pesticides. If aquatic amphibian testing will be required for pesticide risk assessment nevertheless, acute tests with the model organism *X. laevis* and the application of an appropriate uncertainty factor might be a promising approach.

Keywords: Aquatic toxicity, Species sensitivity distribution, *Oncorhynchus mykiss*, Fungicide, Insecticide, Viticulture

Background

Latest reports of the International Union for the Conservation of Nature [24] suggest that 41% of all amphibian species are threatened. In the EU, 21 of 89 amphibian species are listed as critically endangered, endangered, or vulnerable for their global conservation status. Next to habitat loss and fragmentation, diseases, and climate change, exposure to anthropogenic pollutants such as agrochemicals is hypothesized to be one of the main causes of amphibian decline [1036]. Although debates about the assessment of potential effects of pesticides on amphibians are ongoing, amphibians are not yet considered in the current EU environmental risk assessment (ERA) of pesticides. Currently, the risk assessment of potential effects on aquatic amphibian life stages relies on use of data of standard test organisms such as fish [27]. Therefore, it is important to assess the sensitivity of amphibians to pesticides and compare their sensitivity to other taxa such as the standard test species rainbow trout (*Oncorhynchus mykiss*). Several meta-studies and critical reviews have already compared the sensitivities of larval amphibian stages and fish to environmental toxicants. For example, Birge et al. [7], Fryday and Thompson [16] and Weltje et al. [37] determined in general lower sensitivities of amphibians than fish. However, the majority of these comparisons is based on pesticides that are no longer commonly used in the EU (e.g., DDT, atrazine, carbaryl or chlorinated pesticides like chlorpyrifos and lindane). Moreover, many of these studies focus on model species not native to Europe such as North American species or (sub-)tropical species like the African clawed frog (*Xenopus laevis*). *X. laevis* is often used as model species for amphibians [520] because it is easy to culture and handle in laboratory and there is a wide knowledge of its developmental biology [14]. However, there are few comparative toxicity data for *X. laevis* relative to other amphibian species. Several studies have found that *X. laevis* is more tolerant to environmental pollutants than other amphibian species [72030]. In addition, the European common frog (*Rana temporaria*) was described as more sensitive than *O. mykiss* and *X. laevis* towards heavy metals and industrial effluents [7]. It remains unclear whether the sensitivity of tadpoles of the aquatic species *X. laevis* to pesticides is also protective for semi-aquatic species native in Europe [27].

These shortcomings question the assumption that standard test species such as *O. mykiss* and *X. laevis* might be protective surrogates also for Central European amphibian

species. Therefore, the aim of the present study was (i) to assess the sensitivity of larvae of eight native Central European species towards commercial formulations of the two pesticides folpet and indoxacarb and (ii) to compare the sensitivity of these native species with the sensitivity of *X. laevis* using experimentally derived sensitivities of *X. laevis*, and to the sensitivity of *O. mykiss* using values from the literature.

Materials and methods

Pesticide formulations

The tests were performed with commercial formulations of the viticulturally used fungicide folpet (Folpan® 500 SC, 38–42% a.i., hereafter Folpan) and insecticide indoxacarb (Avaunt® EC, 15.84% a.i., hereafter Avaunt). Viticulture is one of the most pesticide-intensive cultures in Central Europe and both pesticides are the most common German viticultural fungicide and insecticide, respectively [35]. Formulations were used instead of technical grade active ingredients because it represents a more realistic scenario as non-target organisms such as amphibians are exposed to these products, not merely to active ingredients. Moreover, previous studies showed that formulation co-formulants may affect the toxicity to amphibians [832].

Folpet is an organochlorine phthalimide and used as a protective, broad-spectrum fungicide against leaf spot diseases in grapevines. The acute aquatic toxicity leads to 96-h LC50 values of 0.233 mg folpet/L [4] and 0.256 mg Folpan/L for *O. mykiss* [1]. The oxadiazine indoxacarb is effective against early life stages of Lepidoptera, Orthoptera, Hemiptera and Coleoptera via contact or ingestion with a 96-h LC50 > 0.17 mg indoxacarb/L [4]] and 7.0 mg Avaunt/L for *O. mykiss* [9].

Test species

In total, nine amphibian species were tested. Besides the standard laboratory species *X. laevis* (Daudin, 1802), we investigated the Central European native species common toad *Bufo bufo* (Linnaeus, 1758), green toad *Bufotes viridis* (Laurenti, 1768), natterjack toad *Epidalea calamita* (Laurenti, 1864), common tree frog *Hyla arborea* (Linnaeus, 1758), common spadefoot toad *Pelobates fuscus* (Laurenti, 1768), water frog *Pelophylax* sp. (Fitzinger, 1843), agile frog *Rana dalmatina* (Fitzinger, 1839), and common frog *Rana temporaria* (Linnaeus, 1758). Between April 2018 and May 2019, parts of three to five egg clutches of each native test species except for *E. calamita* were collected from breeding ponds in South Germany (Additional file 1: Table S1). *E.*

calamita individuals were found only as early hatched tadpoles. Native species were collected from non-agricultural breeding ponds to reduce the potential of evolutionary adaption to pesticides [1123] except for *B. viridis* because no populations from non-agricultural sites were available. A definite differentiation between *P. ridibundus*, *P. lessonae* and the hybridized form of both (*P. esculentus*) was not possible. Thus, we refer to *Pelophylax* sp. as a water frog species. *X. laevis* were obtained from the in-house culture of Eurofins Agroscience Services EcoChem GmbH (Niefern-Öschelbronn, Germany). Information about the threat status and used habitats of the selected test species in Germany and Europe can be found in the supplementary material (Additional file 2: Table S2).

Housing and experiments were performed in a climate chamber (WK 19′/ + 15–35, Weiss Technik GmbH, Reiskirchen, Germany) with a 16:8-h light:dark rhythm at 21 ± 1 °C. The collected egg clutches were distributed to aerated 15-L aquaria (32 × 24 × 20 cm) filled with FETAX medium [12]. Medium renewal took place every other day. Developmental stages were assigned according to Gosner [17] for native species and Nieuwkoop und Faber [26] for *X. laevis*. Native species were tested in the non-feeding, freshly hatched larval Gosner stage (GS) 20 because Adams and Brühl [2] showed higher sensitivity of GS20 in comparison to the commonly used GS25 of *R. temporaria* to the fungicide folpet. *E. calamita* had to be tested in the free-swimming GS25 because no embryos were found in nature and individuals had already developed to GS25 after the performance of range-finding tests. *X. laevis* was tested in the freshly hatched Nieuwkoop–Faber stage (NF) 41–45.

Acute toxicity tests

The study design was derived from the OECD TG 203 (Fish, Acute Toxicity Test; [29]) and TG 202 (Daphnia sp. Acute Immobilisation Test [28]). To provide guidance on the final test concentrations, 48-h range-finding tests with three concentrations of each pesticide formulation and a control group with three replicates of one individual were performed for each species. In the final tests, 96-h median lethal concentrations (LC50) of each species were determined in a static dose–response set-up with six concentrations (Table 1). Tests were conducted in 1.7 L glass jars filled with 1 L test solution prepared with FETAX medium. Per concentration, five replicates with five randomly chosen individuals were used resulting in 150 tadpoles per test. No feeding took place during the tests and dead tadpoles were removed every 24 h. After 96 h, the experiments were terminated, mortality was determined, and all surviving tadpoles were euthanized with a 0.1% buffered MS-222

solution. Concentrations at the beginning of the test and at test termination were analysed for indoxacarb ($n = 9$, SI document 1). Due to the rapid degradation of folpet in aquatic environments (DT50 = 1.2 h at pH 7, DT50 = 1 min at pH 9; values extracted from Agriculture and Environment Research Unit of the University of Hertfordshire 2013), concentration measurements would have not increased the explanatory power of this study as no reliable concentrations would have been generated.

Table 1 Intended test concentrations of Folpan (38-42% folpet) and Avaunt (15.84% indoxacarb) used in acute toxicity tests

Pesticide	Species	Test concentration (mg formulation/L)					
		1	2	3	4	5	6
Folpan	*Bufo bufo*	0	1.0	1.5	2.0	2.5	3.0
	Bufotes viridis	0	0.7	0.9	1.1	1.3	1.5
	Epidalea calamita	0	0.5	1.0	1.5	2.0	2.5
	Hyla arborea	0	1.5	1.7	1.9	2.1	2.3
	Pelobates fuscus	0	1.2	1.3	1.4	1.5	1.6
	Pelophylax sp.	0	0.3	0.4	0.5	0.6	0.7
	Rana dalmatina	0	1.2	1.5	1.8	2.1	2.4
	Rana temporaria	0	0.4	0.5	0.6	0.7	0.8
	Xenopus laevis	0	1.4	1.7	2.0	2.3	2.6
Avaunt	*Bufo bufo*	0	0.4	0.6	0.8	1.0	1.2
	Bufotes viridis	0	2.0	2.5	3.0	3.5	4.0
	Epidalea calamita	0	1.0	1.2	1.4	1.6	1.8
	Hyla arborea	0	2.0	2.5	3.0	3.5	4.0
	Pelobates fuscus	0	2.5	3.0	3.5	4.0	4.5
	Pelophylax sp.	0	8.8	9.0	9.2	9.4	9.6
	Rana dalmatina	0	2.2	2.5	2.8	3.1	3.4
	Rana temporaria	0	1.5	2.0	2.5	3.0	3.5
	Xenopus laevis	0	7.0	7.2	7.4	7.6	7.8

Statistical analyses

For statistical analyses the software R for Windows [33], Version 4.0.2) was used. The extension package "drc" [34] was used to fit a dose–response model for each amphibian species and pesticide formulation (Additional file 3: Table S3). Candidate models were log-normal functions (LN.2, LN.3, LN.4), log-logistic functions (LL.2, LL.3u, LL.4, LL.5), and Weibull-functions (W1.2, W1.3, W1.4, W2.2, W2.3, W2.4). Models were selected by using Akaike information criterion (AIC). Afterwards, LC50 values were calculated for each species and formulation. All amphibian species were ordered from most to least sensitive towards the pesticide formulations and pairwise comparisons via LC50 ratio test after Bonferroni correction [38] were performed to assess significant differences in sensitivity between species. If 95% lower and upper confidence intervals of the calculated differences did not include zero, the differences were judged statistically significant (Additional file 4: Table S4).

Generation of species sensitivity distributions and derivation of risk assessment parameters

Species sensitivity distributions (SSDs) can be used as an ecotoxicological tool for the derivation of quality criteria in ERA. They represent the variation in species sensitivities to a specific contaminant by a statistical distribution function of responses for a sample of species [31]. SSDs were computed with the package "fitdistrplus" [13] for both formulations using the 96-h LC50 values of the European amphibian species to compare their sensitivities. By fitting a suitable statistical distribution to the data, the concentration at which 5% of species were affected by the formulations (HC5, hazard concentration) was derived as the 5th percentile of the SSDs [31]. To determine whether *O. mykiss* and *X. laevis* are suitable surrogate species for European amphibians, the 96-h LC50 literature values for *O. mykiss* and the determined 96-h LC50 values for *X. laevis* were compared to the European amphibian. Moreover, regulatory acceptable concentrations (RACs) which are determined in the current tier 1 risk assessment for fish by dividing their LC50 values by an assessment factor of 100 were compared to the calculated HC5 values derived from the native amphibian SSDs.

Results

Amphibian sensitivities

No mortality was observed in any of the control groups. Sensitivity towards the pesticide formulation Folpan varied between all tested amphibian species in the decreasing order *Pelophylax* sp. > *Rana temporaria* > *Bufotes viridis* = *Epidalea calamita* = *Pelobates fuscus* > *Hyla arborea* > *Rana dalmatina* = *Xenopus laevis* = *Bufo bufo* (">" denotes significant difference, "=" denotes no difference; SI Table 4). Native amphibian sensitivities ranged from 0.44 – 2.19 mg Folpan/L a sensitivity range of a factor of five (Figure 1, Table 2). The HC5 of Folpan for European amphibians was determined to be 0.52 mg Folpan/L.

Sensitivities towards Avaunt decreased in the order *Bufo bufo* > *Epidalea calamita* > *Rana dalmatina* > *Hyla arborea* = *Rana temporaria* = *Bufotes viridis* > *Pelobates fuscus* > *Xenopus laevis* > *Pelophylax* sp. and ranged from 0.86 – 9.43 mg Avaunt/L, thus revealing eleven-fold sensitivity differences (Figure 2, Table 2). The SSD of Avaunt revealed a HC5 of 0.83 mg Avaunt/L.

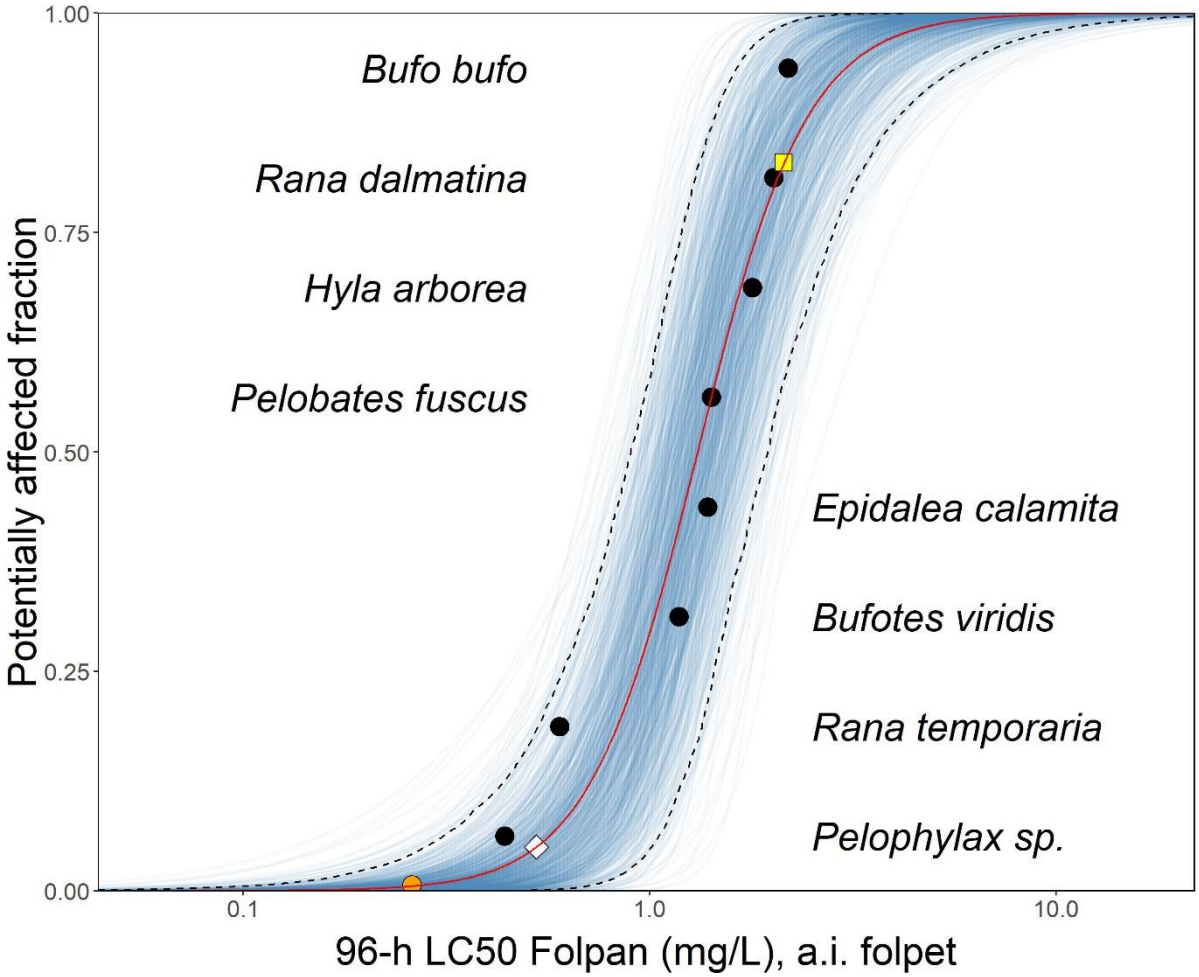

Fig. 1 Species sensitivity distribution of Folpan (38–42% a.i. folpet) calculated from European amphibian sensitivities (red line). Black filled circles denote 96-h LC50 values of European amphibian species. For better comparison, the determined 96-h LC50 values of *Xenopus laevis* (yellow filled square) and the 96-h LC50 literature value of *Oncorhynchus mykiss* (orange filled circle) were included for the formulation Folpan. Species names are aligned in ascending order from bottom to top on the same y-axis coordinate as their respective LC50 value. Dashed lines represent parametric bootstrap 95% confidence intervals (1000 iterations) of native amphibian species. Blue lines display all parametric bootstrap samples of native amphibian species. White filled diamond marks the HC5 value for native amphibian species

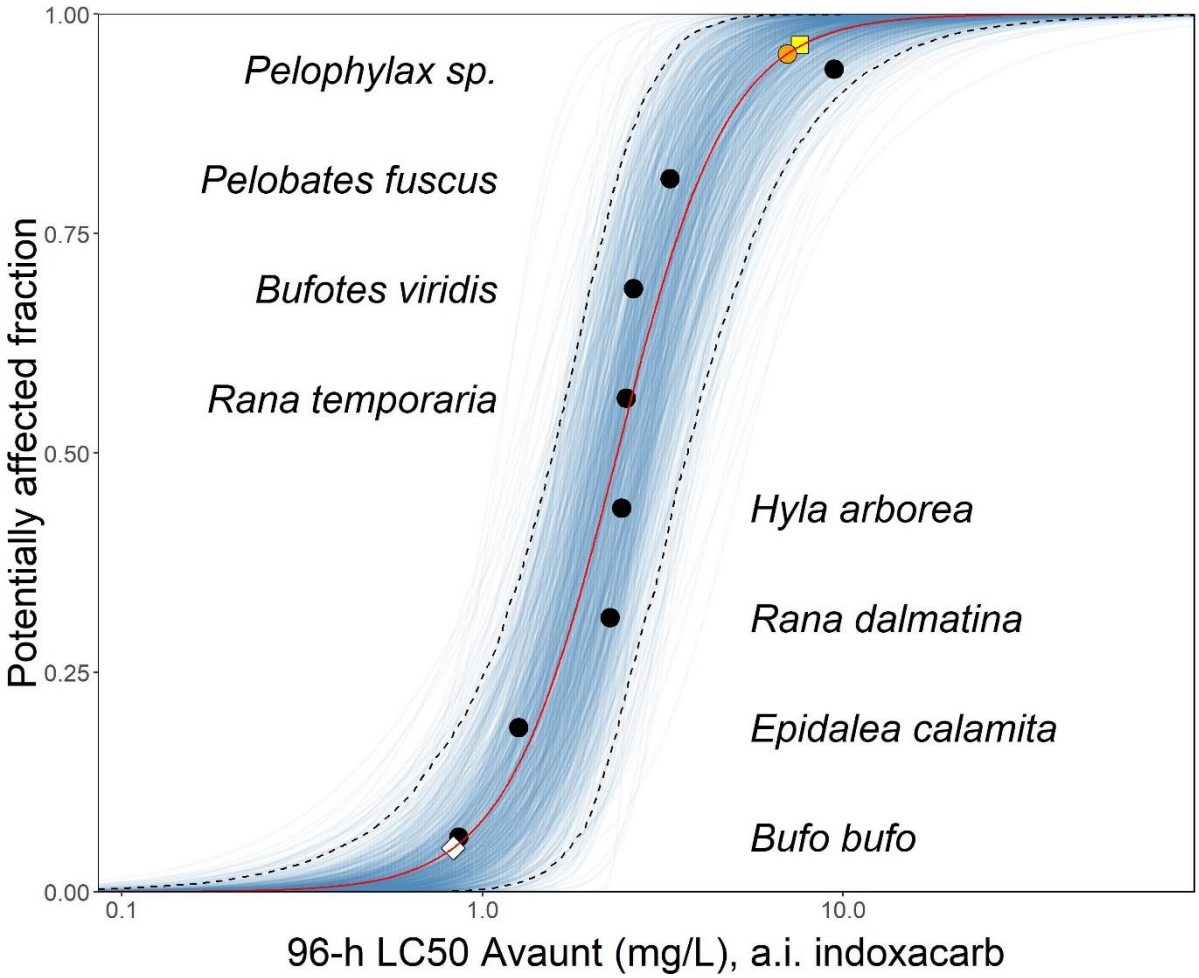

Fig. 2 Species sensitivity distribution of Avaunt (15.84% a.i. indoxacarb) calculated from European amphibian sensitivities (red line). Black filled circles denote 96-h LC50 values of European amphibian species. For better comparison, the determined 96-h LC50 values of *Xenopus laevis* (yellow filled square) and the 96-h LC50 literature value of *Oncorhynchus mykiss* (orange filled circle) were included for the formulation Avaunt. Species names are aligned in ascending order from bottom to top on the same y-axis coordinate as their respective LC50 value. Dashed lines represent parametric bootstrap 95% confidence intervals (1000 iterations) of native amphibian species. Blue lines display all parametric bootstrap samples of native amphibian species. White filled diamond marks the HC5 value for native amphibian species

Table 2 Formulation LC50 values of studied amphibian species and literature LC50 values of *Oncorhynchus mykiss*

Species	Folpan LC50 [mg/L]	Avaunt LC50 [mg/L]
Bufo bufo	2.19	0.86
Bufotes viridis	1.18	2.62
Epidalea calamita	1.39	1.26
Hyla arborea	1.79	2.43
Pelobates fuscus	1.42	3.31
Pelophylax sp.	0.44	9.43
Rana dalmatina	2.02	2.26
Rana temporaria	0.60	2.50
Xenopus laevis	2.14	7.59
Oncorhynchus mykiss	0.256	7.0

Comparison of European amphibians to O. mykiss and X. laevis

Xenopus laevis was the second most tolerant species towards Folpan with a nearly five times lower sensitivity than the most sensitive amphibian species *Pelophylax* sp. *O. mykiss* showed the highest sensitivity towards Folpan (0.256 mg Folpan/L) with a 1.8-fold higher sensitivity than *Pelophylax* sp. For the formulation Avaunt, *X. laevis* was the second most tolerant amphibian species with a nearly nine times lower sensitivity than the most sensitive species *B. bufo*. The formulation LC50 of 7.0 mg Avaunt/L for *O. mykiss* leads to the second lowest sensitivity with an 8.1-fold lower sensitivity than the most sensitive amphibian species *B. bufo*.

Discussion

Suitability of standard test species like *O. mykiss* and the model amphibian species *X. laevis* as surrogate species for European native amphibian species was questioned by EFSA [27]. For interpretation of the results, it needs to be taken into account that only one population of each species was investigated, thus representing limited genetic variability. Moreover, individuals were collected from natural ponds. Thus, exposure and adaptation to potentially present pesticides cannot be excluded. Because *B. viridis* embryos were collected from an agricultural pond, tadpoles might have been less sensitive than individuals from non-agricultural sites [1123]. As the water frog species was not identifiable definitely, our study might not cover sensitivity variations within the water

frog species complex. It further needs to be mentioned that *E. calamita* was the only species tested in the feeding stage GS25. However, sensitivity differences to earlier stages are expected to be in the same range because *R. temporaria* tadpoles in GS20 showed a LC50 of 1.01 mg/L and tadpoles in GS25 a LC50 of 1.22 mg/L for a folpet formulation [2]. Therefore, we expected sensitivity differences between GS20 and GS25 for *E. calamita* to be neglectable compared to differences to other species and decided to consider the sensitivities of *E. calamita* in our comparisons. However, as stage dependent toxicity such as potential starvation stress might occur, the results for *E. calamita* need to be considered with caution.

Our study shows that no general conclusion can be drawn for amphibian sensitivity differences and the use of surrogate species for all pesticide classes because amphibian sensitivities varied between the tested pesticides with 5- to 11-fold differences. The detected sensitivity differences may be due to different modes of action and physiological properties of the species because the fungicide folpet acts as cell division inhibitor of many microorganisms with a multi-site activity [4] whereas the insecticide indoxacarb is a sodium channel blocker that acts via contact and stomach action [4]. Interestingly, the most sensitive species towards Folpan *Pelophylax* sp. was the least sensitive species towards Avaunt. On the other hand, *B. bufo* was the most sensitive species towards Avaunt but the least sensitive species towards Folpan indicating a complete reversal of these two species in sensitivity. Also other studies found contrasting results. Harris et al. [19] observed a lower sensitivity of *B. americanus* embryos towards the fungicide mancozeb than *R. pipiens* embryos, but reverse results for the insecticide endosulfan. These different results show that amphibian sensitivity differences cannot be defined only by family or pesticide class.

In the present study, *X. laevis* was five to nine times more tolerant than the most native amphibian species. In contrast to our results, comparisons of the sensitivities of *X. laevis* to *Pelophylax ridibundus* revealed *X. laevis* tadpoles to be more sensitive towards the insecticide methidathion and the herbicide glyphosate [18]. However, based on our findings, *X. laevis* can be used as surrogate for acute risk assessments of Central European aquatic amphibian stages when applying a minimum uncertainty factor of at least 9 that covers higher sensitivities of the tested native species.

Sensitivity of *O. mykiss* towards Folpan seems to be in the same range as for the tested amphibian species thus indicating a suitable surrogate for aquatic stages of European

amphibian species. The LC50 of 7.0 mg Avaunt/L for *O. mykiss* indicates an 8.1-fold lower sensitivity than the most sensitive species *B. bufo*. Assuming an aquatic tier 1 risk assessment, the recommended uncertainty factor of 100 for fish acute toxicity tests [15] leads to RACs of 2.56×10^{-3} mg Folpan/L and 0.07 mg Avaunt/L. These RACs would cover the sensitivity of all tested amphibian species. They also cover the determined HC5 values which are 200 and 12 times higher than the determined RACs. Thus, considering the assessment factor of 100 which is used in the tier 1 risk assessment, the assumption of Birge et al. [7], Fryday und Thompson [16], and Weltje et al. [37] that acute toxicity data of standard fish species (here *O. mykiss*) are suitable to cover the sensitivity of aquatic amphibian stages was confirmed for the investigated European amphibian species in an early hatchling stage for two current-use pesticides. It needs to be noted that all tests were carried out under laboratory conditions at stable temperatures of 21 °C. Temperature and other environmental conditions are co-stressors which might change the amphibian sensitivity [625]. Additionally, it remains unclear whether fish and amphibians are similarly sensitive to formulation co-formulants [2122]. Especially terrestrial stages seem to be very sensitive to co-formulants in formulations [38] which might be particularly toxic to the permeable skin of terrestrial amphibians.

Conclusions

For the first time the present study assessed aquatic sensitivity differences of Central European amphibian species in comparison to standard test organisms such as *O. mykiss* and *X. laevis*. The results of our study support the notion of preceding reviews that acute toxicity data generated using standard aquatic test species meet the requirements for acute aquatic amphibian risk assessment after the application of the assessment factor of 100. If aquatic amphibian testing will be required for pesticide risk assessment nevertheless, test methods with the model organism *X. laevis* considering the application of a reliable uncertainty factor might be a promising approach. Substantial research on interaction of temperature and pesticide stress, formulation and terrestrial toxicity is still necessary to derive standardized acute toxicity tests and a protective ERA for amphibians.

Abbreviations

a.i.	Active ingredient
DT	Dissipation time
ERA	Environmental risk assessment
EU	European Union
HC	Hazard concentration
IUCN	International Union for the Conservation of Nature
LC	Lethal concentration
SSD	Species sensitivity distribution

Supplementary Information

The online version contains supplementary material available at https:// doi.org/ 10. 1186/ s12302- 021- 00491-1.

Acknowledgements

We thank Haiying Xia for her laboratory assistance and Philipp Uhl for his support in the statistical analyses. The authors are grateful for the provision of *Xenopus laevis* embryos by Valentin Mingo of Eurofins Agroscience Services EcoChem GmbH.

Authors' contributions

EA: conceptualization, methodology, investigation, formal analysis, writing—original draft preparation. CL: conceptualization, writing—reviewing and editing. APR: pesticide residue analysis, writing—reviewing and editing. CAB: conceptualization, writing—reviewing and editing. All authors read and approved the final manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL. EA was financed by the Ph.D. scholarship program of the German Federal Environmental Foundation. CL was financed by the Deutsche Forschungsgesellschaft (DFG-TH 1807-2).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The experiments and withdrawal of animals were approved by the Federal Investigation Office (Landesuntersuchungsamt, Koblenz, Germany) to § 8a of the German law for animal welfare (license number 23 177-07/G18-20-009), the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license number 42/553-254/455-18) and the Regierung der Oberpfalz (Regensburg, license number ROP-SG55.1-8646.4-1-1115-3).

Consent for publication

Not applicable.

Competing interests

EA is involved in the private sector. However, the conceptualization, methodology and formal analysis of the present paper were performed when all authors were affiliated with the University of Koblenz-Landau. Therefore, we see no conflict of interest arising from this involvement for the present submission.

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Supporting information of scientific publication 1

SI Document 1. Pesticide residue analysis.

To verify the pesticide concentrations during the 96-h test period, it was planned to quantify the amount of a.i. of both pesticide formulations at the start and end of the test period. To investigate the general degradation of indoxacarb in the Avaunt samples, three control replicates, three replicates of the lowest test concentration (0.4 mg Avaunt/L = 63 µg indoxacarb/L) and three replicates of the highest test concentration (9.6 mg Avaunt/L = 1521 µg indoxacarb/L) were analyzed at the beginning (t0) and the end of the test (t96).

Folpet rapidly degrades in aquatic environments (DT50 = 1.2 h at pH 7, DT50 = 1 min at pH 9; values extracted from the Pesticide Properties DataBase, Agriculture and Environment Research Unit of the University of Hertfordshire) to its major metabolite phthalimide. Due to the rapid degradation of folpet, concentration measurements neither make sense from an analytical point of view nor would it increase the value and explanatory power of the study.

Chemicals and reagents

LCMS grade water, acetonitrile and methanol were purchased and used for the preparation of samples and instrument mobile phase solutions. Formic acid, ammonium formate and an indoxacarb analytical standard were purchased from Sigma-Aldrich.

Sample Preparation

A working stock of indoxacarb, with a concentration of 400 mg/L in acetonitrile, was prepared and used for all further dilutions. Calibration standards with the concentrations 0 µg/L, 2.5 µg/L, 5.0 µg/L, 10.0 µg/L, and 16.0 µg/L were prepared in FETAX medium. Low concentration samples (63 µg/L) were diluted 1:10 and high concentration samples (1521 µg/L) were diluted 1:100 in FETAX medium before measurement. Standards and samples were filtered with a 0.2 µm PTFE filter before analyzing.

HPLC-ESI-MS/MS Analysis

Chromatographic separations were carried out on an Agilent 1260 Infinity II liquid chromatography system (Santa Clara, CA, USA) using a ZORBAX Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm), purchased from Agilent technologies. The separation conditions were as follows: Solvent (A) H_2O/MeOH (98:2) and solvent (B) H_2O/MeOH (2:98), with both solutions containing 4 mM ammonium formate and 0.1% formic acid. The gradient elution was as follows: initial conditions 60% A, 2-5 minutes 0% A, 5.1-8.5 minutes 60% A, 1-minute post run delay. Injection volume was 1 µL, the mobile phase flow rate was set to 0.4 mL/min and

the column temperature was maintained at 45 °C during the analysis. The HPLC system was connected to an Agilent 6495C Triple Quadrupole LC/MS with an electrospray ionization source (ESI). The source conditions were as follows: gas temperature: 250 °C, gas flow: 11 L/min, nebulizer pressure: 38 psi, sheath gas temperature: 350 °C, sheath gas flow: 12 L/min, capillary voltage: 3000 V and nozzle voltage: 0 V. Three multiple reaction monitoring (MRM) transitions were acquired in positive mode for the identification and quantification of indoxacarb, these were: 528 -> 203 (as quantifier), 528 -> 149.9 and 528 -> 293; with collision energies of 40, 25 and 9, respectively. Data generated by the mass spectrometer were analyzed using MassHunter quantitative data analysis software (Version 10.0.707.0). The retention time of indoxacarb was 5.25 min.

Quality assurance information

For the quality assurance, all three MRM transitions were required for the positive identification of indoxacarb. In addition, the ratio between the qualifiers' and the quantifiers´ transitions were not allowed to deviate by more than ±30% from the ratio in the corresponding matrix matched standards. Retention times of all sample peaks were within ±2.5% of the retention times of the peaks in the corresponding calibration standards. The linear five-point matrix matched calibration curve, with a R^2 value of 0.99, was used for quantification.

SI Document 1 Table 1. Intended and calculated concentrations of indoxacarb standards

Intended conc. (µg/L)	Area	Calc. conc. (µg/L)
0	0.0	0.0
2.5	15561.1	3.2
5.0	20228.3	4.2
10.0	49875.7	10.7
16.0	73033.6	15.7

SI Document 1 Table 2. Intended and measured concentrations of indoxacarb samples at test initiation (t0) and test termination (t96)

Replicate	Intended conc. (µg/L)	Meas. conc. t0 (µg/L)	Percentage of t0 of intended conc. (%)	Meas. conc. t96 (µg/L)
1	0.0	0.0	-	0.0
2	0.0	0.0	-	0.0
3	0.0	0.0	-	0.0
1	63.0	48.0	76	19.0
2	63.0	49.0	78	21.0
3	63.0	54.0	86	17.0
		Mean	**80**	
1	1520	1270	84	460
2	1520	1300	86	480
3	1520	1300	86	490
		Mean	**85**	

SI Table 1. Withdrawal locations of studied European test species.

Species	**Common name**	**Coordinates of withdrawal ponds (WGS84)**
Rana temporaria	Common frog	49.25475, 7.96182
Bufo bufo	Common toad	49.25475, 7.96182
Hyla arborea	Common tree frog	49.258425, 8.406791
Rana dalmatina	Agile frog	49.256260, 8.404287
Bufotes viridis	European green toad	49.317, 8.12906
Pelobates fuscus	Common spadefoot toad	49.310415, 8.314917
Pelophylax sp.	Pool frog, Edible frog or hybrid of both	49.264016, 8.438084
Epidalea calamita	Natterjack toad	49.532500, 11.994333

SI Table 2. Artificial terrestrial and aquatic habitats of examined amphibian species.

Extinction risk classification in Europe according to IUCN and Status in Germany [1]

NA = Not applicable, NE = Not Endangered, WL = Early Warning List, LC = Least Concern, NR = Near Threatened, VU = Vulnerable, EN = Endangered, CR = Critically Endangered

Species	Common name	Status in Europe	Status in Germany	Artificial terrestrial and aquatic habitats according to IUCN
Rana temporaria [2]	Common frog	LC	NE	Arable Land | Pastureland | Plantations | Rural Gardens | Urban Areas Water Storage Areas | Aquaculture Ponds | Excavations | Wastewater Treatment Areas | Seasonally Flooded Agricultural Land
Bufo bufo [3]	Common toad	LC	NE	Arable Land | Pastureland | Plantations | Rural Gardens | Urban Areas Water Storage Areas | Excavations | Irrigated Land and Irrigation Channels | Canals and Drainage Channels, Ditches
Hyla arborea [4]	Common tree frog	LC	EN	Arable Land | Pastureland | Rural Gardens | Urban Areas

				Ponds | Irrigated Land and Irrigation Channels | Canals and Drainage Channels, Ditches
Rana dalmatina [5]	Agile frog	LC	NE	No terrestrial Ponds | Canals and Drainage Channels, Ditches
Bufotes viridis [6]	European green toad	LC	EN	Arable Land | Pastureland | Rural Gardens | Urban Areas Ponds | Irrigated Land and Irrigation Channels | Canals and Drainage Channels, Ditches
Pelobates fuscus [7]	Common spadefoot toad	LC	EN	Arable Land | Pastureland | Rural Gardens | Urban Areas Ponds | Excavations | Irrigated Land and Irrigation Channels | Seasonally Flooded Agricultural Land | Canals and Drainage Channels, Ditches
Pelophylax sp. [8]	Pool frog, Edible frog or hybrid of both	LC	NE	Arable Land | Pastureland | Rural Gardens | Urban Areas Water Storage Areas | Ponds | Aquaculture Ponds | Excavations | Wastewater Treatment Areas | Seasonally Flooded Agricultural Land
Epidalea calamita [9]	Natterjack toad	LC	WL	Arable Land | Pastureland | Rural Gardens

				Ponds | Excavations | Irrigated Land and Irrigation Channels | Seasonally Flooded Agricultural Land | Canals and Drainage Channels, Ditches
Xenopus laevis [10]	African clawed frog	NA	NA	Plantations Urban Areas Subtropical/Tropical Heavily Degraded Former Forest Water Storage Areas | Ponds | Aquaculture Ponds | Excavations | Irrigated Land and Irrigation Channels | Seasonally Flooded Agricultural Land | Canals and Drainage Channels, Ditches

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SI Table 3. Model specification on which each 96-h LC50 value is based.

Candidate models were log-normal functions (LN.2, LN.3, LN.4), log-logistic functions (LL.2, LL.3u, LL.4, LL.5), and Weibull-functions (W1.2, W1.3, W1.4, W2.2, W2.3, W2.4).

Pesticide	Species	Function	R function
Folpan	*Bufo bufo*	Two-parameter Weibull	W2.2
	Bufotes viridis	Five-parameter log-logistic	LL.5
	Epidalea calamita	Two-parameter Weibull	W2.2
	Hyla arborea	Two-parameter Weibull	W1.2
	Pelobates fuscus	Two-parameter Weibull	W1.2
	Pelophylax sp.	Three-parameter Weibull	W1.3
	Rana dalmatina	Two-parameter Weibull	W2.2
	Rana temporaria	Two-parameter Weibull	W1.2
	Xenopus laevis	Three-parameter Weibull	W1.3
Avaunt	*Bufo bufo*	Three-parameter log-logistic	LL.3u
	Bufotes viridis	Three-parameter Weibull	W1.3
	Epidalea calamita	Two-parameter Weibull	W1.2
	Hyla arborea	Three-parameter Weibull	W1.3
	Pelobates fuscus	Two-parameter Weibull	W1.2
	Pelophylax sp.	Three-parameter Weibull	W1.3
	Rana dalmatina	Two-parameter Weibull	W1.2
	Rana temporaria	Log-normal	LN.2
	Xenopus laevis	Three-parameter Weibull	W1.3

SI Table 4. Contingency table of estimated difference of LC50 via CI ratio testing.

Pesticide	Comparison	Estimate	SE	LCI	UCI
Folpan	*Pelophylax* sp. – *Rana temporaria*	-0,16	0.01	-0.19	-0.13
	Rana temporaria – Bufotes viridis	-0.58	0.10	-0.85	-0.31
	Bufotes viridis – Epidalea calamita	-0.21	0.10	-0.49	0.07
	Epidalea calamita – Pelobates fuscus	-0.03	0.02	-0.09	0.03
	Pelobates fuscus – Hyla arborea	-0.37	0.01	-0.41	-0.33
	Hyla arborea – Rana dalmatina	-0.23	0.03	-0.32	-0.14
	Rana dalmatina – Xenopus laevis	-0.12	0.11	-0.43	0.19
	Xenopus laevis – Bufo bufo	-0.05	0.11	-0.36	0.26
Avaunt	*Bufo bufo – Epidalea calamita*	-0.40	0.02	-0.46	-0.34
	Epidalea calamita – Rana dalmatina	-1.00	0.04	-1.11	-0.89
	Rana dalmatina – Hyla arborea	-0.17	0.05	-0.31	-0.03
	Hyla arborea – Rana temporaria	-0.07	0.07	-0.25	0.11
	Rana temporaria – Bufotes viridis	-0.12	0.06	-0.29	0.05
	Bufotes viridis – Pelobates fuscus	-0.69	0.02	-0.75	-0.63
	Pelobates fuscus – Xenopus laevis	-4.28	0.18	-4.78	-3.78
	Xenopus laevis – Pelophylax sp.	-1.84	0.19	-2.35	-1.33

Appendix A.2 – Scientific publication 2

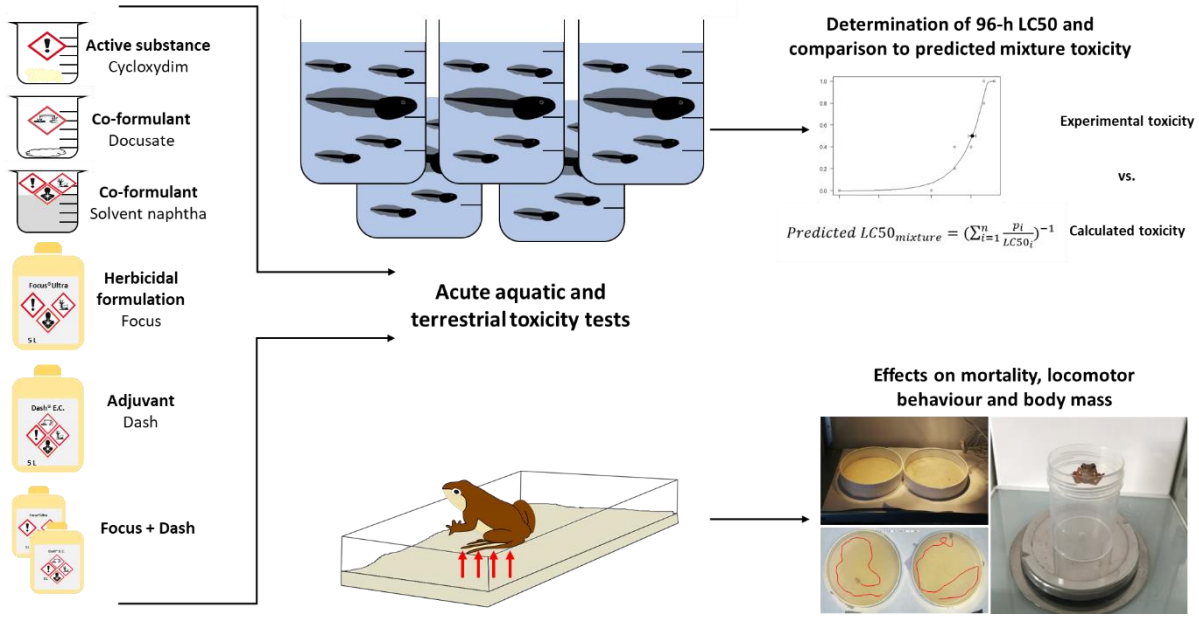
Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to European common frogs (*Rana temporaria*)

Elena Adams, Verena Gerstle, Tobias Schmitt, Carsten A. Brühl

Highlights

- Formulation toxicity depends on co-formulants and the addition of adjuvants
- The terrestrial exposure to these can lead to lethal and sublethal effects
- Aquatic toxicity does not predict terrestrial toxicity to amphibians
- Formulation toxicity needs special consideration in the amphibian risk assessment

Graphical abstract



Abstract

While pesticides are generally recognized as contributing to amphibian declines, there is a lack of knowledge about effects of co-formulants that are present in pesticide formulations and adjuvants which are mixed with these formulations. Since aquatic and terrestrial stages of amphibians can be exposed to these substances, adverse effects cannot be excluded. We investigated acute aquatic and terrestrial effects of the herbicide formulation Focus® Ultra, its active substance cycloxydim, its co-formulants solvent naphtha and docusate as well as the stabilizing adjuvant Dash® E.C. on larval and juvenile *Rana temporaria*. Aquatic toxicity was determined as 96-h LC50 values. Cycloxydim was the least toxic and solvent naphtha the most toxic substance of the formulation. The addition of Dash® E.C. increased the formulation toxicity substantially. Terrestrial toxicity was determined as lethal effects after a 48-h exposure to contaminated soil with 100% of the recommended field rate (FR) and as sublethal effects after the exposure to 10% of the recommended FR. The exposure to solvent naphtha and docusate at 100% FR led to mortalities of 42-100% probably due to their inhalation toxicity and dermal as well as eye irritation, respectively. Cycloxydim, Focus® Ultra and Dash® E.C.

did not lead to any mortality. Sublethal effects on juvenile locomotor activity (i.e. moved distance) were observed for cycloxydim and the combined exposure of Focus® Ultra and Dash® E.C. Juvenile body masses declined significantly for all substances except for cycloxydim.
The present results show that aquatic sensitivity does not predict terrestrial sensitivity. It was shown that pesticide toxicity for amphibians can highly depend on the presence and amount of co-formulants and added adjuvants. Therefore, substances included in pesticide formulations which are known to be toxic by inhalation or harmful to eyes or skin should be specifically considered in the environmental risk assessment for amphibians.

Keywords
Amphibian, Pesticide, Focus® Ultra, Tadpole, Behaviour, Mortality

1 Introduction

Pesticide formulations are mixtures of one or more active substances and co-formulants (EU, 2009). While pesticides are generally recognized as contributing to amphibian declines (Stuart et al., 2004), there is a lack of knowledge of all chemicals present in pesticide formulations to which aquatic and terrestrial stages of amphibians are exposed. Following European Union terminology, co-formulants are substances or preparations which are used in pesticide formulations or adjuvants (EU, 2009). They are neither active substance nor synergists or safeners, which enhance the activity of the active substance or reduce phytotoxic effects of pesticides on certain plants, respectively. Co-formulants that have been proven to induce harmful effects on animal health or unacceptable effects on the environment shall not be accepted for inclusion in pesticide formulations (EU, 2009). Adjuvants are substances or preparations consisting of co-formulants and are placed on the market separately to be mixed with a pesticide formulation before application to enhance efficacy (EU, 2009). Thus, co-formulants and adjuvants are introduced in addition to active substances to the environment, potentially exerting adverse effects. However, the necessary investigations to disentangle toxicity of formulation components are complicated due to limited data access regarding proprietary information. Scientists of independent research institutions do not frequently have access to the composition of pesticide formulations and not all chemicals comprised in formulations are necessarily described in the safety data sheets.

Several studies indicate that co-formulants can be toxic themselves or enhance the toxicity of pesticide formulations to amphibians (e.g. Brühl et al., 2013; Hooser et al., 2012; Wagner et al., 2015). Amphibians might be highly sensitive to co-formulants and adjuvants because of their

biphasic life-cycle and thus a combined aquatic and terrestrial exposure. Moreover, their permeable skin that enables water regulations (Wells, 2007) also facilitates the uptake of larger molecules such as pesticides through the dermal barrier (Kaufmann and Dohmen, 2016; Quaranta et al., 2009). Several studies observed increased dermal absorption of pesticide formulations and co-formulants in comparison to their active substances alone (Baynes and Riviere, 1998; Brand and Mueller, 2002; Reifenrath, 2007). Therefore, amphibians are especially threatened due to their high dermal uptake capacity.

In addition to enhanced absorption, increased toxicity of formulations or toxicity of co-formulants themselves were observed in several studies on lethal and sublethal level of amphibians. Increased mortality of aquatic stages after exposure to formulations of the insecticide permethrin (Boone, 2008) and the herbicide glyphosate (Howe et al., 2004) has been observed in comparison to the active substances alone. Lethal formulation effects of the fungicide pyraclostrobin and glyphosate have been observed for early terrestrial amphibian stages (Brühl et al., 2013; Relyea, 2005). Effects of formulations were also observed on a sublethal level such as effects on the aquatic development (Howe et al., 2004) and *in vitro* neurotoxic effects (Swann et al., 1996). These studies show that knowledge about the toxicity of active substances does not per se allow a prediction about the effect of pesticide formulations.

Another example for formulation effects was investigated by Wagner et al. (2015) who determined a significantly higher mortality and malformation rate of embryos and early-stage larvae of the African clawed frog (*Xenopus laevis*) after exposure to the herbicide formulation Focus® Ultra in comparison to exposure to the active substance cycloxydim alone. However, effects of this formulation, its active substance and its co-formulants on aquatic and terrestrial stages of European amphibian species remain unstudied. One of the co-formulants, solvent naphtha, has already been shown to potentially increase the toxicity of pyraclostrobin fungicide formulations on juvenile *Rana temporaria* at environmentally relevant concentrations (Brühl et al., 2013), thus suggesting the possibility for similar increased toxicity of Focus® Ultra compared to the active substance.

Currently, the risk assessment of pesticide effects on aquatic amphibian life stages is assumed to be covered by the use of data of surrogate species (Weltje et al., 2013) such as the standard temperate fish species rainbow trout (*Oncorhynchus mykiss*). Although dermal pesticide exposure of postmetamorphic juveniles and adults is highly likely (Lenhardt et al., 2015), this pathway is not yet considered in the environmental risk assessment of pesticides and ecotoxicological studies investigating the sensitivity of terrestrial amphibian stages to

pesticides are considerably rare (e.g., Adams et al., 2020; Brühl et al., 2013; Leeb et al., 2020; Relyea, 2005). This scarcity might be due to ethical restrictions regarding animal testing and the unavailability of standardized test guidelines. Moreover, it is not known whether the effects of active substances, co-formulants, adjuvants and pesticide formulations are comparable between aquatic and terrestrial amphibian stages.

Based on these uncertainties, the aims of the present study were (i) to investigate the aquatic toxicity differences between the herbicide formulation Focus® Ultra, its active substance cycloxydim, its two co-formulants solvent naphtha and docusate as well as the adjuvant Dash® E.C, that is part of the combination package Focus® Aktiv-Pack, (ii) to compare the experimentally determined formulation and package toxicity to predicted toxicity values based on a concentration addition model, and (iii) to determine lethal and sublethal effects of environmentally relevant concentrations of each substance to terrestrial juvenile amphibians. As the European common frog (*Rana temporaria*) is one of the most widespread amphibian species (Sillero et al., 2014) and it has been investigated in previous aquatic and terrestrial amphibian toxicity tests (e.g., Adams et al., 2020; Adams and Brühl, 2020; Brühl et al., 2013) we used it as surrogate for European anuran species.

2 Material and methods

2.1 Test substances

The combination package Focus® Aktiv-Pack including the herbicidal pesticide formulation Focus® Ultra (hereafter Focus) and the adjuvant Dash® E.C. (hereafter Dash; both manufactured by BASF SE, Ludwigshafen, Germany) was purchased from a local distributor. Ingredients of Focus according to the safety data sheet are the active substance cycloxydim (10.8%) and the co-formulants docusate (dioctyl sodium sulfosuccinate, < 5% according to BASF, 2018b, 2.4% w/w according to Wagner et al., 2017), and solvent naphtha (< 60% according to BASF, 2018b, 50% w/w according to Wagner et al., 2017, 47.2% w/w according to BVL, 2015). The formulation is applied once per season with a maximum field application rate of 5.0 L/ha in 150-300 L water/ha (BASF, 2018a; BVL, 2015). Fish LC50 values for the formulation, its co-formulants and adjuvant are given in Table 2.

The active substance cycloxydim was purchased as technical grade standard (≤ 100% purity, Merck KGaA, Darmstadt, Germany). Cycloxydim is a cyclohexenone belonging to the HRAC-group A (Herbicide Resistance Action Committee, www.hracglobal.com), which inhibits acetyl-CoA carboxylases in sensitive plants leading to a decreased fatty acid synthesis in

plastids and membrane formation. The co-formulant docusate was purchased by Merck KGaA as sodium salt (≤ 100% purity, Darmstadt, Germany). Docusate is used in medicine as laxative and stool softening agent due to its characteristic as surfactant, allowing water to pass intestine membranes by decreasing their surface tension (Brunton et al., 2018). Solvent naphtha, also known as Solvesso, is a fraction of aromatic hydrocarbons that is generated during the distillation of high temperature coal tar or petroleum. It was purchased by DHC Solvent Chemie GmbH (Mülheim a. d. Ruhr, Germany) as Hydrosol A200 ND that consists of C10-aromatic hydrocarbons with a low content of naphthalene (<1%). The adjuvant Dash is used as an additive to stabilize the efficacy of herbicides and fungicides. For this, the adsorption and wetting behaviour on plant surfaces is optimized by decreasing the pH and surface tension of the spray solution. It is applied with a maximum application volume of 1.0 L/ha (BASF, 2020).

2.2 Animal collection and husbandry

In March 2019, we collected parts of egg clutches of *R. temporaria* from a forest pond in the Palatinate forest in Southwest Germany (49.25475 N, 7.96182 E, WSG84). The pond was expected to be uncontaminated because of its distance to any agricultural area. Water samples analysed in the course of another study (Adams et al., 2021) revealed no pesticide residues. The eggs were kept in aerated aquaria (32 × 24 × 20 cm) filled with filtered tap water (0.2 µm Supor, Pall Corporation, Port Washington, USA) in a laboratory with a 16:8-h light:dark cycle at 21 + 1°C. Water renewal took place every other day. As soon as the tadpoles reached the free-swimming and feeding Gosner stage (GS) 25 (Gosner, 1960), they were fed ad libitum on a daily basis with commercially available rearing food (Sera Micron, Sera GmbH, Heinsberg, Germany) until metamorphosis. Juveniles were kept in terraria (32 × 24 × 20 cm) filled with moisturized forest soil, moss, leaves and a water supply. Every other day, juveniles were fed ad libitum with *Drosophila melanogaster* and *D. hydei* obtained from an in-house culture.

2.3 Acute aquatic toxicity tests

Aquatic acute toxicity tests were performed in a climate chamber (WK 19´/+15-35, Weiss Technik GmbH, Reiskirchen, Germany) with a 16:8-h light:dark cycle at 21 + 1°C. Tests were performed with the non-feeding hatchling stage GS20 as this tadpole stage was shown to be most sensitive (Adams and Brühl, 2020). For concentration range finding, 48-h tests with three treatment concentrations of each chemical and a control group with three replicates of one individual per species were performed to provide guidance on the final test concentrations. Final tests were performed as 96-h tests to allow comparison to the 96-h LC50 values for fish. For

each chemical (cycloxydim, docusate, solvent naphtha, Focus, Dash, Focus + Dash), five treatment groups and one control group with five replicates of five individuals (150 randomly selected tadpoles per test) were examined in 1.7 L glass jars filled with 1 L test solution prepared with FETAX medium (Table 1). Next to a control group with FETAX medium, an additional ethanol control group was added because cycloxydim had to be pre-dissolved in 0.001% (v/v) ethanol. Solvent naphtha was tested as a water accommodated fraction, for which the test solutions were stirred for 24 hours with a magnetic stirrer to break up the oil into small droplets that mix more easily with the FETAX medium before introducing the test individuals. A slow stirring was continued until test termination to ensure the presence of solvent naphtha in the water phase. pH-values of all test concentrations were measured using a WTW multiparameter MultiLine Multi 340i and a WTW SenTix pH-electrode (WTW, Weilheim, Germany). No feeding took place during the exposure period and dead tadpoles were removed every 24 hours. To determine 96-h median lethal concentrations (LC50), mortalities were assessed after 96 hours. After test termination, tadpoles were euthanized using a 0.1% buffered MS-222 solution.

Table 1. Nominal concentrations of test substances used in aquatic 96-h acute toxicity tests

Substance	Test concentration [mg/L]					
	1	2	3	4	5	6
Cycloxydim	0	20.0	40.0	60.0	80.0	100
Docusate	0	60.0	62.0	64.0	66.0	68.0
Solvent naphtha	0	5.0	7.5	10.0	12.5	15.0
Focus	0	26.0	27.0	28.0	29.0	30.0
Dash	0	4.0	4.2	4.4	4.6	4.8
Focus + Dash	0	2.2	2.4	2.6	2.8	3.0

2.4 Dermal soil exposure tests

Terrestrial soil exposure tests were performed in a laboratory at 21 + 1°C with a 16:8-h light:dark cycle. A study length of 48 hours was chosen to prevent juveniles from dehydration. Freshly metamorphosed juveniles (seven to ten days old) were kept randomly and individually in clear, lockable plastic terrariums (22.5 × 16.5 × 7 cm, Braplast, Bergheim, Germany) filled with 250 g artificial soil that consisted of 70% industrial sand (particle diameter: 50-200 µm; Euroquartz, Dorsten, Deutschland), 20% kaolin clay (Carl Roth, Karlsruhe, Germany), and 10% sphagnum peat (sieved through 2 mm mesh; Florafort, Floragard, Oldenburg, Germany).

Lenhardt et al. (2015) determined that up to 17% of a reproducing population of the German amphibian species *Bombina bombina* can encounter a herbicide application during bare soil stage in maize. At the leaf development stage with 25% plant interception, about 12% of the *B. bombina* population coincided with herbicides on maize fields. Therefore, a worst-case scenario with 100% of the maximum recommended field rate (FR) was tested. To increase the environmental relevance and to consider pesticide exposure mitigation by interception of crops, a second test using 10% of the maximum recommended FR was performed. For logistical reasons, 10% FR and 100% FR treatments were not tested in parallel, but within the same week. 100% FR were applied with an application volume of 5.0 L Focus/ha and 1.0 L Dash/ha in 200 L water/ha. Since no exact amounts of solvent naphtha and docusate are provided in the safety data sheet of Focus, and due to the varying information according to Wagner et al. (2017) and BVL (2015), 60% and 50% of solvent naphtha (600 mg/L and 500 mg/L) as well as 5% and 2% of docusate (50 mg/L and 20 mg/L) were applied. Cycloxydim was only tested for the 10% FR tests because it was not soluble within the specified limit of solvent use for toxicity testing (0.01%, OECD, 2019) in the 100% FR tests. In the 10% FR tests, 0.5 L Focus/ha and 0.1 L Dash/ha were applied. To reduce vertebrate testing, only the highest concentration for solvent naphtha (6%, 60 mg/L) and docusate (0.5%, 5 mg/L) were tested in the 10% FR tests. Solvent naphtha spray solutions were mixed for six hours with a magnetic stirrer before use. For each treatment group (control, cycloxydim, docusate, solvent naphtha, Focus, Dash, Focus + Dash) twelve individual replicates were tested. To keep vertebrate testing at a minimum, six control and six solvent controls (0.01% ethanol) were used in the 10% FR tests. Consequently, 84 and 72 individuals were used for the 100% FR and 10% FR tests, respectively.

Treatment and control solutions were prepared in filtered tap water. pH-values of all spray solutions were measured using a WTW multiparameter MultiLine Multi 340i and a WTW SenTix pH-electrode (WTW, Weilheim, Germany). Before application of treatment and control solutions, the soil of each terrarium was pre-wetted with tap water (40 mL/box) to prevent dehydration of juveniles during the test period. The solutions were applied by using a laboratory spray application system (Try Spray Cabinet, Schachtner Gerätetechnik, Ludwigsburg, Germany) with singular nozzles (TeeJet TP80). Juveniles were placed in the terraria two hours after application for 48 hours and were not fed during the exposure period. Mortality of juveniles was assessed after 48 hours. To investigate sublethal effects in the 10% FR tests, juveniles were weighed prior to test initiation and after the exposure period to calculate the relative body mass decline. Moreover, their locomotor behaviour after 48 hours was assessed.

Juveniles were filmed individually for ten minutes after an acclimatization period of three minutes in a circular arena (glass dish with a diameter of 20 cm and height of 5 cm) using eight camera modules (SC15-1, Kuman Ltd., Shenzhen, China) connected to single-board computers (Raspberry Pi 3 Model B, Raspberry Pi Foundation, Cambridge, United Kingdom). The video tracking software EthoVision XT (Noldus Information Technology, 2017) was used to analyse the total distance each juvenile moved. After test termination, juveniles were euthanized using a 0.1% buffered MS-222 solution.

2.4 Statistical analyses

For statistical analyses the software R for Windows (R Core Team, 2020 ,Version 4.0.2) was used. For all statistical tests, the criterion for significance was set to $\alpha = 0.05$. The extension package "drc" (Ritz and Streibig, 2005) was used to fit a dose-response model for each tested substance (SI Table A1). Candidate models were log-normal functions (LN.2, LN.3, LN.4), log-logistic functions (LL.2, LL.3u, LL.4, LL.5), and Weibull-functions (W1.2, W1.3, W1.4, W2.2, W2.3, W2.4). Models were selected based on Akaike information criterion (AIC). After the calculation of 96-h LC50 values for each component, they were compared via LC50 ratio test after Bonferroni correction as described by Wheeler et al. (2006). For this, asymptotic-based 95% confidence intervals were calculated using the method "delta" as interval settings. If 95% lower and upper confidence intervals of the calculated differences did not include zero, the differences were judged statistically significant (SI Table A2).

In a review of the European Commission (Kortenkamp et al., 2009), the use of a concentration addition (CA) model was proposed as most relevant concept of mixture toxicity. Therefore, the predicted aquatic mixture toxicities for the combination of cycloxydim, solvent naphtha and docusate as well as for the combination of Focus and Dash were calculated according to equation 1 and compared to the measured LC50 values of Focus and the combination of Focus and Dash, respectively.

$$Predicted\ LC50_{mixture} = \left(\sum_{i=1}^{n} \frac{p_i}{LC50_i}\right)^{-1} \qquad \text{Equation 1}$$

where:

n = number of mixture components

i = index from 1 to n mixture components

p_i = the i^{th} component as a relative fraction of the mixture composition

$LC50_i$ = LC50 of component i

Afterwards, equation 2 was used to calculate the model deviation ratio (MDR) according to EFSA (2013). The MDR can be used to counter-check the calculated and measured mixture toxicity of the formulation as well as the combination of the formulation and the adjuvant and to determine if the components act more (i.e. synergistically) or less (i.e. antagonistically) than expected by the CA. The observed and calculated mixture toxicities are considered in agreement if the MDR is between 0.2 and 5. If the MDR is higher than 5, a synergistic mixture toxicity is indicated. An MDR below 0.2 indicates an antagonistic mixture toxicity.

$$MDR = \frac{LC50_{mixture,calculated}}{LC50_{mixture,measured}}$$ Equation 2

Body mass decline and distance moved data of the terrestrial juveniles were checked for normality and homogeneity of variances. Tukey´s method was used to identify and remove outliers ranging above and below the 1.5 × IQR (Kannan Senthamarai et al., 2015). Differences in moved distance between treatment groups were compared using analysis of variance (ANOVA) with consecutive post-hoc Tukey´s test. Non-parametric Kruskal-Wallis test with consecutive Dunn´s test was applied for the body mass decline data. p-values were adjusted using the Benjamini-Hochberg method.

2.5 Animal Welfare

The experiments were approved by the Federal Investigation Office of Rhineland-Palatinate (Landesuntersuchungsamt, Koblenz, Germany) to § 8a of the German law for animal welfare with the approval number 23 177-07/G18-20-009, and the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license number 42/553-254/455-18).

3 Results

3.1 Aquatic toxicity

Since no mortality was observed in either the control or ethanol groups, the results of the water control and solvent control were combined as recommended by Green and Wheeler (2013). Because the limit of solubility was reached for cycloxydim and no mortality was observed at the highest tested concentration of 100 mg/L, no dose-response model could be fitted to the data and no LC50 was obtained. The determined LC50 values of the other substances ranged from 2.44 – 62.4 mg/L (Table 2) and were significantly different from each other (SI Table A2): The active substance cycloxydim was least toxic (LC50 > 100 mg cycloxydim/L). The

two co-formulants led to LC50 values of 62.4 mg docusate/L and 10.2 mg solvent naphtha/L. The exposure to the formulated product Focus resulted in a LC50 of 29.4 mg Focus/L which is six times higher than the LC50 of the adjuvant Dash (4.56 mg Dash/L) and twelve times higher than the LC50 of the combined exposure to Focus and Dash as an equitoxic mixture (2.44 mg mixture/L). Measured pH-values of the test solutions were 7.6 (control), 7.5 (cycloxydim), 7.5 (docusate), 7.4 (solvent naphtha), 7.5 (Focus), 7.2 (Dash), and 7.3 (Focus + Dash).

To allow predictive mixture toxicity calculations, the LC50 of cycloxydim was set to the highest tested concentration of 100 mg/L with a ratio of 10.8% of the formulations. Since the safety data sheet of Focus only provides imprecise content ratios, the calculations were conducted using ratios of 50% and 60% for solvent naphtha as well as 2% and 5% for docusate. Therefore, two difference predicted LC50 values were obtained for each co-formulant (Table 3). The different ratios of docusate did not influence the outcome, but solvent naphtha ratio changes did. The predicted LC50 values ranged from 16.5 (60% solvent naphtha, 2% and 5% docusate) to 19.9 mg/L (50% solvent naphtha, 2% and 5% docusate) which is 32 – 44% lower than the measured LC50 of Focus. The predicted mixture toxicity of Focus and Dash in an equitoxic mixture led to a predicted LC50 of 7.90 mg/L, which is three times higher than the measured LC50 value for the combined exposure of Focus and Dash. The calculated MDR values (Table 3) ranged from 0.56 to 3.24, thus indicating neither a synergistic nor an antagonistic effect.

Table 2. Measured aquatic 96-h LC50 values of investigated with 95% confidence intervals and standard error for *Rana temporaria* and literature 96-h LC50 values for fish. Because no mortality of 50% for cycloxydim was achieved, no dose-response model could be fitted to the respective data. The determined LC50 values were significantly different from each other

Substance	LC50 [mg/L]	Lower 95% CI [mg/L]	Upper 95% CI [mg/L]	Standard error [mg/L]	Fish LC50 [mg/L]
Cycloxydim	> 100	NA	NA	NA	> 220 [1]
Docusate	62.37	61.99	62.75	0.19	49 [2]
Solvent naphtha	10.22	9.56	10.88	0.32	2.0 [3]
Focus	29.40	27.58	31.22	0.89	20.4 [4]
Dash	4.56	4.30	4.83	0.13	22 [5]
Focus + Dash	2.44	2.43	2.45	0.01	NA

[1] Determined for Oncorhynchus mykiss (Agriculture and Environment Research Unit of the University of Hertfordshire 2013)

[2] Determined for Danio rerio (Sigma-Aldrich 2018).

[3] Determined as LL50 (lethal loading rate of water accommodated fractions; water accommodated fractions are media prepared via low energy mixing of a poorly soluble test material such as oil; Aurand und Coelho 2005) for O. mykiss (DHC 2018).

[4] Determined for O. mykiss (BASF 2018).

[5] Determined for O. mykiss (BASF 2016).

Table 3. Predicted aquatic mixture LC50 values and calculated model deviation ratio (MDR) for the combination of the active substance cycloxydim and the co-formulants solvent naphtha and docusate with different content ratios as well as for the combination of Focus and Dash

	SN:D ratio 60:5 and 60:2	SN:D ratio 50:5 and 50:2	Focus:Dash ratio 50:50
Predicted mixture LC50 [mg/L]	16.5	19.9	7.9
Measured mixture LC50 [mg/L]	29.4	29.4	2.44
MDR	0.56	0.68	3.24

SN = solvent naphtha, D = docusate

3.2 Terrestrial mortality

After the exposure to 100% FR of the substances, no mortality was observed for the control, Focus, Dash and both in combination. The dermal exposure to 5% and 2% of docusate lead to 67% and 42% mortality. Solvent naphtha led to 100% mortality after 60% and 40% exposure. After the exposure to soil contaminated with 10% of the recommended FR, no juveniles died. pH-values of all spray solutions ranged from 7.1 (10% FR Dash) to 7.6 (control) except for 100% FR solvent naphtha (4.3), 100% FR Focus (6.2), 100% FR Dash (2.1), and 100% FR Focus and Dash (2.3).

3.3 Locomotor behaviour

Because the control and solvent control data did not differ significantly, the results were combined as recommended by Green and Wheeler (2013). The distance moved by juveniles in ten minutes after the 48-h exposure to soil contaminated with 10% FR of the investigated components ranged from 88.9 cm (cycloxydim) to 167.8 cm (Focus, SI Table A3). The exposure induced statistically significant differences to the control ($df = 6$, $F = 5.99$, $p < 0.001$). Significant declines were observed for the active substance cycloxydim (67% reduction, $p < 0.01$) and the combined exposure of Focus and Dash (63% reduction, $p < 0.01$) in comparison to the control (Figure 1, SI Table A4). Moreover, animals in the cycloxydim treatment group and the combined Focus and Dash treatment group moved significantly less than the individuals of the Focus treatment group (89% and 84% reduction, respectively, $p < 0.001$).

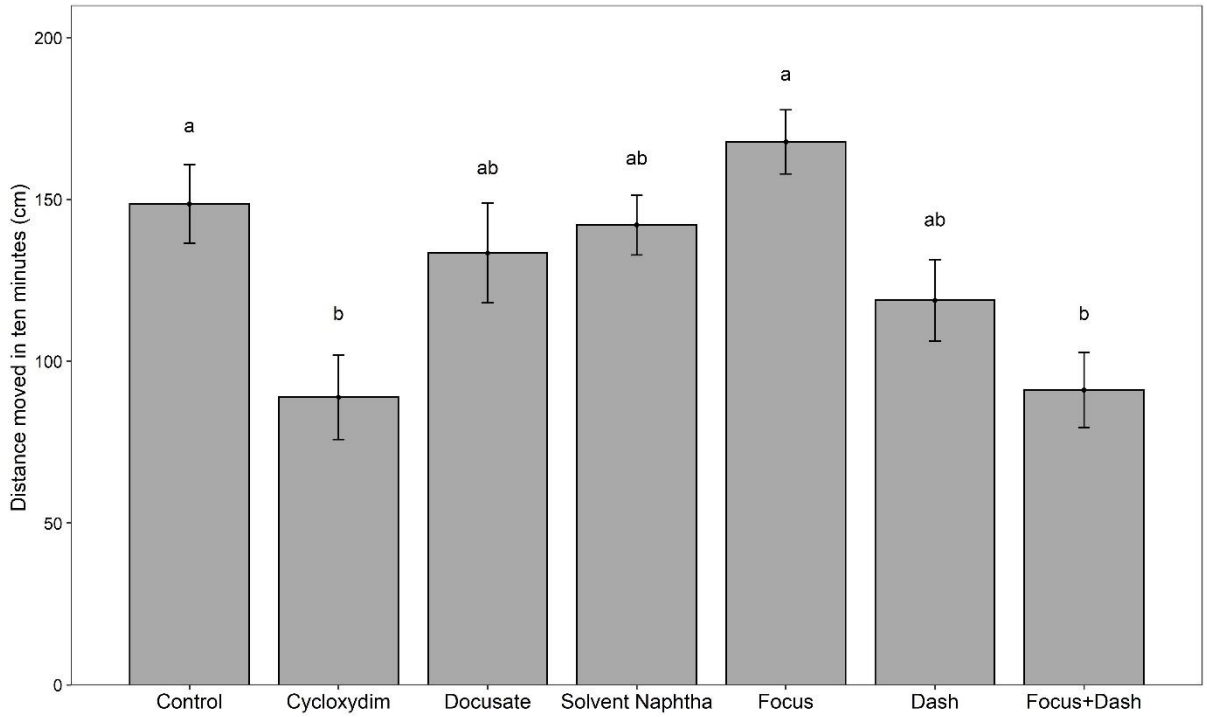


Figure 1. Total mean moved distance ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$).

3.4 Body mass decline

The 48-h dermal exposure to soil contaminated with 10% of the FR of the tested substances led to mean mass declines ranging from 8.8% (control) to 15.1% (Dash, SI Table A5). The Kruskal-Wallis test revealed statistically significant body mass declines ($X^2 = 32.74$, $df = 6$, $p < 0.001$). Exposure to the different substances induced significantly increased body mass declines in juveniles for docusate ($p < 0.01$), solvent naphtha ($p < 0.05$), Focus ($p < 0.001$), Dash ($p < 0.001$) and the combination of Focus and Dash ($p < 0.05$) when compared to the control group (Figure 2, SI Table A6).

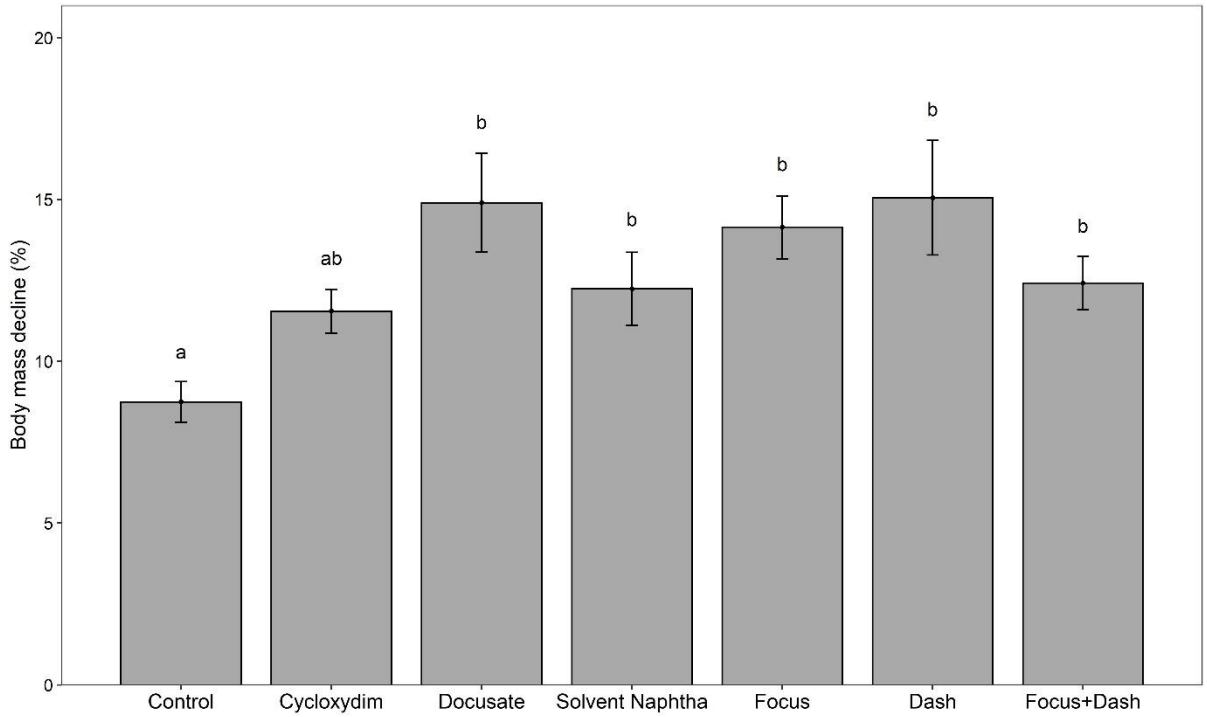


Figure 2. Relative mean body mass decline ± standard error of juvenile *Rana temporaria* after 48-h exposure to 10% of the field rate of the investigated substances. Letters represent statistically significant differences ($p < 0.05$).

4 Discussion

4.1 Acute aquatic toxicity

Due to the low acute fish toxicity (Table 2), the active substance was expected to have a low toxicity to *R. temporaria* tadpoles which was confirmed by a LC50 > 100 mg/L. As indicated by the fish LC50, docusate did not lead to high aquatic toxicity in tadpoles. Solvent naphtha was expected to show high toxicity due to the low fish LC50 and its GHS (Globally Harmonized System of Classification and Labelling of Chemicals) classification as harmful for aquatic organisms, which was confirmed by the lowest determined LC50 value of the formulation components. Due to the higher fish LC50 values of Focus and Dash, a moderate toxicity was expected for both substances. This assumption was confirmed for Focus as the exposure of tadpoles to the formulation led to a 1.4-times higher LC50 than for fish. However, the exposure to Dash led to a LC50 of 4.56 mg/L, which is five times lower than the fish LC50. Because no LC50 value of the combination of Focus and Dash is given in the safety data sheet, a comparison to amphibian sensitivity is not possible.

Wagner et al. (2015) determined a four-times lower toxicity of cycloxydim (96-h LC50 of 4.0 mg/L) than of Focus (96-h LC50 of 0.9 mg/L) to early larval stages of *X. laevis*. These findings

indicate that *X. laevis* tadpoles were 33-times and 25-times more sensitive than *R. temporaria* tadpoles towards the formulation and the active substance, respectively. Thus, the results confirm the lower toxicity of the active substance in comparison to the formulation but also emphasize the consideration of species sensitivity differences. As the pH-values of the aquatic test solutions were all in the range of 7.2 – 7.6, they are not the reason for different observed toxicities, but rather the systemic toxicity of the tested compounds.

4.2 Acute aquatic environmental risk assessment considerations

Except for Dash, all determined amphibian LC50 values were lower than the fish LC50 values. Despite the five times lower LC50 for Dash, for acute environmental risk assessment purposes, the fish LC50 values would cover the determined amphibian sensitivity after the application of the recommended uncertainty factor of 100 for acute aquatic toxicity (EFSA, 2013).

Depending on the co-formulant contents, the predicted aquatic mixture toxicity of the formulation components was 1.8-times and 1.5-times lower than the measured LC50 for Focus. Thus, the predicted Focus LC50 value would cover the aquatic amphibian sensitivity. However, it needs to be considered that the content of solvent naphtha clearly affects the toxicity of the formulation because of its low measured LC50 but also because of its decreasing effect on the predicted, calculated LC50. Therefore, co-formulants exhibiting high toxicities themselves should be considered with a priority in the risk assessment of pesticide formulations. These results show that the calculation of the predicted mixture toxicity might be a good tool to assess formulation toxicity to aquatic stages of amphibians. However, the predicted aquatic mixture LC50 of the combination of Focus and Dash was three times higher than the measured LC50, thus underestimating the toxicity of combined exposure of the formulation and the adjuvant. This underestimation might be due to the additional toxicity of Dash that consists of a non-ionic surfactant (ethoxylated alcohol), nonfatty acid methyl ester, and oleic acid (BASF, 2016). Lewis (1992) investigated toxicity trends for mixtures containing surfactants and pesticides. They found that it is difficult to generalize or predict synergistic, antagonistic or additive toxicities of these mixtures. The interaction of surfactants and other chemicals has been found to affect different functions and multiple cellular response targets, which generate a complex cascade of events in organisms that cannot be easily summarized (Wei et al., 2009). Therefore, an uncertainty factor should be applied in the environmental risk assessment of formulations and adjuvants to ensure no unacceptable risk to amphibians.

In the Central European risk assessment, a predicted environmental concentration (PEC) in surface water of 4.6 mg Focus/L was determined for spray drift (BVL, 2015). Moreover, the PEC considered for entry via runoff is 0.22 µg cycloxydim/L, and for drainage 0.51 µg cycloxydim/L or 0.17 µg cycloxydim/L in autumn/winter or spring/summer, respectively. As these environmentally relevant concentrations are considerably lower than the determined LC50 values, no acute amphibian toxicity is expected for the active substance and the formulation in the environment.

4.3 Terrestrial toxicity

The mortality results after exposure to soil contaminated with 100% FR show that the sole exposure to the co-formulants docusate and solvent naphtha is most toxic as it led to 42-100% mortality whereas no mortality was observed in other treatment groups. Because the juvenile frogs were not directly oversprayed and test solutions were likely buffered by the artificial soil, the different pH values of the test solutions probably did not have an acute effect on amphibian survival. A more probable reason for lethality might be an effect on the juveniles' skin. As docusate acts as a surfactant, it decreases the surface tension and thus the barrier of membranes, allowing penetration of water and therefore potentially also of the test solution into the body (Brunton et al., 2018). This membrane modification might have led to docusates GHS classification as "causes serious eye damage" and "causes skin irritation". The high acute toxicity of solvent naphtha might be caused by its GHS classification as "may be fatal if swallowed and enters airways". On the one hand, inhalation toxicity is especially relevant for amphibians because of their high respiration rate based on their metabolic rate which is increased due to their poikilothermy (Halsey and White, 2010). On the other hand, the entire skin of terrestrial amphibian stages is a respiratory organ and for small individuals with a high surface-to-volume ratio, skin breathing covers an essential part of respiration (up to 30% of O_2 uptake and 70% CO_2 elimination; Burggren and Moallf, 1984). Thus, adverse effects on lung as well as dermal respiration might be the reason for the high toxicity of solvent naphtha. Interestingly, no mortality was observed after exposure to the formulation including docusate and solvent naphtha. This might either indicate an interaction of compounds in the formulation or altered for example volatility or surfactant properties of the co-formulants in the formulation. Future research should verify concentrations of the co-formulants in soil to allow further interpretation of this finding.

Increased toxicity with increasing solvent naphtha content in pesticide formulations was also indicated by Brühl et al. (2013). They determined 100% mortality of juvenile *R. temporaria*

after direct overspray with a fungicide formulation containing the active substance pyraclostrobin and 67% solvent naphtha. On the contrary, only 20% of the juveniles died after overspray with a pyraclostrobin formulation containing <25% solvent naphtha. These results confirm our findings that solvent naphtha is highly toxic for terrestrial stages of amphibians.

Sublethal effects of formulation co-formulants and adjuvants after the exposure to 10% FR were different than indicated by the determined lethal effects after 100% FR exposure. Moved distance was affected by the exposure to cycloxydim and the combination of Focus and Dash. In this context the non-significant gradual decrease in moved distance by juveniles exposed to Focus, Dash and the combination of Focus and Dash became apparent, indicating an enhanced sublethal toxicity by the addition of the adjuvant Dash. Lower activity might play an important role due to the juveniles´ key role in the dispersal of amphibians (Cushman, 2006). Thus, the reduced activity might further contribute to local amphibian declines. Moreover, it could lead to an impaired predation behaviour as it was observed by Adams et al. (2020) for juvenile *R. temporaria* after exposure to the fungicide folpet. Such an effect might lead to a decreased survival that further impairs overall population survival chances. In general, most studies investigating amphibian behavioural responses to pesticides focus on larval amphibian stages (Sievers et al., 2019) emphasizing the underrepresentation of terrestrial amphibian stages in ecotoxicological studies (Brühl et al., 2011). Most studies investigating pesticide effects on terrestrial stages did not find behavioural alterations (see review of Brühl et al., 2011) that might be due to a lack of standardized methods and endpoints to analyse such responses (Leeb et al., 2020).

Statistically significant body mass declines were observed after the exposure of every test solution except for cycloxydim. A smaller body mass might represent an increased risk of predation and low survivorship at maturity (Berven and Gill, 1983; Smith, 1987). Adams et al. (2020) determined a non-significant overall body mass decline of *R. temporaria* juveniles after 48-h exposure to the fungicide folpet. In contrast, Webber et al. (2010) did not find an effect on the growth of juvenile Great Plains toads (*Anaxyrus cognatus*) after exposure to the insecticide carbaryl. The herbicide atrazine was shown to affect the body mass of Gray tree frogs (*Hyla versicolor*) at metamorphosis (Diana et al., 2000). These results show that effects on body mass also depend on the pesticide that amphibians are exposed to.

The observed declines might have been developed as hydration loss due to irritated or damaged skin caused by the co-formulants. With respect to the great importance of water for amphibians, this hydration loss might indicate stress regarding the osmoregulation and thus affecting vital

functions of the juveniles (Shoemaker and Nagy, 1977). Cusaac et al. (2017) did not find an adverse effect of dehydration on the mortality of terrestrial stages of two North American toad species after exposure to a pyraclostrobin fungicide formulation. In contrast, they determined a reduced mortality in comparison to hydrated toads. This difference might have been attributed to behavioural and physiological adaptations to dehydration in toads (Cusaac et al., 2017). They observed that juvenile toads kept their ventral seat patch elevated to avoid water loss. Furthermore, they observed reduced activity of the dehydrated juveniles, who frequently aggregated in the corners of the aquaria, a behaviour that is consistent with conserving water. A hydrated stratum corneum in the membrane of the investigated juveniles may have been substantially more permeable than dehydrated skin (Trommer and Neubert, 2006). This might be an explanation for the toxicity differences between the test compounds. Moreover, as solvent naphtha and docusate directly affect the amphibian membrane also in the pesticide formulation, cycloxydim alone might have been excluded from any body burden in the single compound exposure.

5 Conclusions

The present study showed that pesticide toxicity in amphibians can highly depend on the presence and amount of co-formulants in formulations and added adjuvants. Because detailed information on formulation composition is difficult to obtain, more information on the types of co-formulants and adjuvants included in or added to pesticide formulations and their fate in the environment is required. The knowledge about the presence of co-formulants and adjuvants would greatly aid in assessing the exposure and potential toxicity for amphibians and other non-target organisms. As adverse effects on the skin of terrestrial amphibian stages might not be identified in aquatic tests, we recommend including the toxicity of co-formulants and adjuvants which are known to be harmful to eye or skin or toxic by inhalation in the toxicity evaluation of pesticides for terrestrial amphibian toxicity. Ultimately, the use of substances such as solvent naphtha in pesticide formulations should be avoided to reduce adverse effects on amphibian populations.

Acknowledgements

We thank Christoph Hoffmann and Thomas Gramm of the Julius Kühn-Institut at Geilweilerhof, Siebelingen for using their laboratory spray application system and the help during application processes. We are grateful to Martin Entling for providing access to the

software Ethovision. Sincere thanks to Arnd Weyers, Bayer CropScience for his valuable comments on the study evaluation.

Funding

This work was supported by the Ph.D. scholarship program of the German Federal Environmental Foundation to E. Adams.

Data availability statement

Data are available by contacting E. Adams (adams@uni-landau.de).

Author contribution statement

Elena Adams: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original draft preparation; Verena Gerstle: Investigation, Writing – Reviewing and Editing; Tobias Schmitt: Investigation, Writing – Reviewing and Editing; Carsten A. Brühl: Conceptualization, Writing – Reviewing and Editing

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Supporting information of scientific publication 2

Supplementary material – Data and results of statistical analyses

Table A1. Model specification on which each aquatic 96-h LC50 value is based (Ritz and Streibig, 2005). Candidate models were log-normal functions (LN.2, LN.3, LN.4), log-logistic functions (LL.2, LL.3u, LL.4, LL.5), and Weibull-functions (W1.2, W1.3, W1.4, W2.2, W2.3, W2.4). Because no mortality of 50% for cycloxydim was achieved, no dose-response model could be fitted to the respective data

Substance	Function
Docusate	W1.2 – Two-parameter Weibull
Solvent naphtha	LL5 – Five-parameter log-logistic
Focus	W1.3 – Three-parameter Weibull
Dash	W1.3 – Three-parameter Weibull
Focus + Dash	W1.2 – Two-parameter Weibull

Table A2. Contingency table of aquatic LC50 comparisons via CI ratio testing (Wheeler et al., 2006). Because no mortality of 50% for cycloxydim was achieved, the comparison via CI ratio testing was not possible for this compound. However, it can be assumed that the LC50 for cycloxydim would significantly differ from the others. If 95% lower and upper confidence intervals of the calculated differences did not include zero, the differences were judged statistically significant

Comparison	Estimate	Std. Error	Lower CI	Upper CI
Docusate – Solvent naphtha	-52.15	0.37	-53.19	-51.10
Docusate – Focus	32.97	0.91	30.43	35.52
Docusate – Dash	57.81	0.23	57.16	58.45
Docusate – Focus + Dash	59.93	0.19	59.40	60.46
Solvent naphtha – Focus	-19.17	0.94	-21.82	-16.53
Solvent naphtha – Dash	5.66	0.34	4.69	6.63
Solvent naphtha – Focus + Dash	7.79	0.32	6.89	8.69
Focus – Dash	24.83	0.90	22.32	27.35
Focus – Focus + Dash	26.96	0.89	24.47	29.45
Dash – Focus + Dash	2.13	0.13	1.76	2.49

Table A3. Moved distances in cm after 48-h terrestrial dermal exposure to 10% of the FR of the tested substances

Treatment group	Mean	Median	Standard deviation	Standard error
Control	148.59	131.02	72.76	12.13
Cycloxydim	88.88	67.92	61.27	13.06
Docusate	133.45	111.12	53.50	15.44
Solvent naphtha	142.11	139.19	43.59	9.29
Focus	167.80	166.93	48.40	9.88
Dash	118.82	103.30	61.73	12.60
Focus + Dash	91.09	88.27	56.77	11.59

Table A4. Test statistics of the post-hoc Tukey test for multiple comparisons of means of the total moved distance

Comparison	Difference	Lower CI	Upper CI	p-adjusted
Cycloxydim – Control	-59.71	-107.61	-11.82	**< 0.01**
Dash – Control	-29.78	-76.41	16.86	0.48
Docusate – Control	-15.14	-74.13	43.85	0.99
Focus + Dash – Control	-57.50	-104.14	-10.87	**< 0.01**
Focus – Control	19.21	-27.43	65.85	0.88
Solvent naphtha – Control	-6.48	-54.37	41.41	0.99
Dash – Cycloxydim	29.94	-22.30	82.17	0.61
Docusate – Cycloxydim	44.58	-18.94	108.09	0.36
Focus + Dash – Cycloxydim	2.21	-50.03	54.45	0.99
Focus – Cycloxydim	78.92	26.69	131.16	**< 0.001**
Solvent naphtha – Cycloxydim	53.23	-0.13	106.59	0.05
Focus + Dash – Docusate	-42.36	-104.93	20.21	0.40
Focus – Docusate	34.35	-28.22	96.92	0.66
Solvent naphtha – Docusate	8.66	-54.85	72.17	0.99
Docusate – Dash	14.64	-47.93	77.21	0.99
Focus + Dash – Dash	-27.73	-78.81	23.36	0.67
Focus – Dash	48.99	-2.10	100.07	0.070
Solvent naphtha – Dash	23.29	-28.94	75.53	0.84
Focus – Focus + Dash	76.71	25.63	127.80	**< 0.001**
Solvent naphtha – Focus + Dash	51.02	-1.21	103.26	0.06
Solvent naphtha - Focus	-25.69	-77.93	26.54	0.76

Significant differences are highlighted in bold.

Table A5. Body mass declines in percent after 48-h terrestrial dermal exposure to 10% of the FR of the tested substances

Treatment group	Mean	Median	Standard deviation	Standard error
Control	8.74	7.60	3.67	0.63
Cycloxydim	11.55	10.75	3.19	0.68
Docusate	14.90	15.61	5.28	1.52
Solvent naphtha	12.24	12.86	5.51	1.12
Focus	14.14	14.78	4.65	0.97
Dash	15.05	14.63	8.31	1.77
Focus + Dash	12.42	12.39	3.77	0.82

Table A6. Test statistics of the post-hoc Dunn test for multiple comparisons of body mass declines

Comparison	Z	p-unadjusted	p-adjusted
Control – Cycloxydim	-2.08	0.037	0.11
Control – Docusate	-3.71	**< 0.001**	**< 0.01**
Control – Solvent naphtha	-3.06	**< 0.01**	**< 0.05**
Control – Focus	-4.25	**< 0.001**	**< 0.001**
Control – Dash	-4.68	**< 0.001**	**< 0.001**
Control – Focus + Dash	-2.63	**< 0.01**	**< 0.05**
Cycloxydim – Docusate	1.88	0.06	0.14
Cycloxydim – Solvent naphtha	-0.84	0.40	0.53
Cycloxydim – Focus	-1.94	0.05	0.14
Cycloxydim – Dash	-2.36	**0.05**	0.06
Cycloxydim – Focus + Dash	-0.52	0.60	0.74
Dash – Docusate	0.10	0.92	0.92
Docusate – Solvent naphtha	1.21	0.23	0.34
Docusate – Focus	0.27	0.79	0.83
Docusate – Focus + Dash	1.42	0.15	0.27
Dash – Solvent naphtha	1.57	0.12	0.22
Dash – Focus	0.44	0.66	0.77
Dash – Focus + Dash	1.81	0.07	0.15
Focus – Solvent naphtha	1.14	0.26	0.36
Focus + Dash – Solvent naphtha	-0.29	0.77	0.85
Focus + Dash – Focus	-1.39	0.16	0.27

Significant differences are highlighted in bold.

Appendix A.3 – Scientific publication 3

Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*)

Elena Adams, Verena Gerstle, Carsten A. Brühl

Abstract

Viticulture is one of the most pesticide-intensive agricultures in Europe, leading to a spatiotemporal overlap of amphibian migration and pesticide applications. Because postmetamorphic, terrestrial amphibian stages are mostly neglected in ecotoxicological studies, we investigated acute effects of viticultural fungicides on juvenile common frogs (*Rana temporaria*). Tadpoles from an uncontaminated pond were placed in enclosures in 8 ponds with an increasing degree of pesticide contamination in southwest Germany to represent different aquatic exposure backgrounds. After metamorphosis, juveniles were exposed to soil contaminated with 50% of the recommended field rates of the fungicides Folpan® 80 water dispersible granule (WDG) and Folpan® 500 suspension concentrate with the same amount of folpet as active ingredient and differing additives. After 48 h, effects on the survival, body mass, and behavior were investigated. No effect of the aquatic exposure background on terrestrial sensitivity could be detected. Acute terrestrial exposure led to mean mortality rates of 14% (13–17%, suspension concentrate) and 60% (17–100%, WDG) and resulted in adverse effects on locomotor activity as well as feeding behavior. Moreover, the results suggest that the toxicity of the 2 tested folpet formulations depends on their additives. Because the identified effects may result in severe impairments and thus in declines of amphibian populations, a more protective risk assessment of pesticides is needed for postmetamorphic amphibians to ensure proper conservation of amphibian populations.

Keywords

Amphibians; Ecotoxicology; Fungicide; Behavioral toxicology; Terrestrial ecotoxicology; Viticulture

INTRODUCTION

Among other stressors, the widespread use of pesticides in agricultural landscapes has been implicated as one of the major drivers for the global amphibian decline (Stuart et al. 2004). In Germany, 51% of the land area is currently used for agriculture (German Environment Agency 2018). Viticulture covers large parts of southern Palatinate in southwest Germany and is one of the most pesticide-intensive types of agriculture in Germany. In these regions, pesticides are applied on average 9.5 times between March and August, of which preventive fungicide applications against mildew diseases contribute an average of 8.6 applications (Roßberg and

Ipach 2015). Within these wine-growing landscapes, rain retention ponds serve as important habitats for pond breeding, biphasic amphibian species (Lenhardt et al. 2017). The spatiotemporal overlap of amphibian migration and pesticide applications likely results in exposure of amphibians at their terrestrial life stages (Lenhardt et al. 2015; Leeb et al. 2020). If pesticides contact their skin, they are taken up dermally (Storrs Méndez et al. 2009; van Meter et al. 2015) because amphibians have a highly permeable skin (Kaufmann and Dohmen 2016), which enables water and ion exchange regulation (Wells 2007) but also facilitates the uptake of larger molecules (Willens et al. 2006), such as pesticides, through the dermal barrier (Storrs Méndez et al. 2009; van Meter et al. 2015).

Several studies have identified sublethal and lethal effects of pesticides on amphibians (see reviews in Mann et al. 2009; Brühl et al. 2011; Fryday and Thompson 2012). Dermal uptake of pesticides can lead to mortality rates of up to 100% after direct overspray with pesticides at environmentally relevant field application rates (Relyea 2005; Belden et al. 2010; Brühl et al. 2013). Besides direct overspray, dermal contact to contaminated soil and vegetation plays an important role in amphibian sensitivity because many compounds remain on environmental surfaces (Silva et al. 2019), which can result in accumulation of pesticides in the organism (van Meter et al. 2015) and in lethal or sublethal effects (Storrs Méndez et al. 2009; Mitchkash et al. 2014). Although the effect of aquatic preexposure to pesticides on the sensitivity of postmetamorphic stages is not clear yet, several aquatic studies have detected genetic adaptation to pesticide exposure and thus decreased sensitivity of tadpoles (Bridges and Semlitsch 2000; Cothran et al. 2013; Hua et al. 2013). However, it has not been proven yet that an aquatic preexposure of tadpoles can change the sensitivity of postmetamorphic, terrestrial stages.

Although dermal pesticide exposure of postmetamorphic, terrestrial amphibian stages (i.e., juveniles and adults) is highly likely in viticultural areas, this pathway is not considered in the environmental risk assessment of pesticides. Given the widespread loss of amphibian populations, it is critical to understand potential risks associated with this pathway. The present study aimed to expand the limited data on dermal pesticide toxicity using realistic worst-case exposure levels via contaminated soil and thereby expand on existing studies that mainly focused on direct pesticide overspray. The objectives of the present study were 1) to investigate sublethal effects, including effects on body mass, moved distance, and feeding behavior, and 2) to evaluate the influence of previous aquatic pesticide exposure during the larval stage on the terrestrial sensitivity of juvenile common frogs, *Rana temporaria* (Linnaeus 1758).

The common frog is listed as “least concern” by the International Union for Conservation of Nature (Kuzmin et al. 2009). It is one of the most common amphibian species in Europe (Sillero et al. 2014) and can often be found in ponds within or near vineyards (Lenhardt et al. 2015). However, according to the Red List of Threatened Species, the common frog is listed as a pre-endangered species in 8 of 16 federal states and as endangered in 2 federal states of Germany (Kühnel et al. 2009). We used juveniles instead of adults because they leave the aquatic habitats between May and June in Germany (Günther 2009), which overlaps with most of the fungicide applications in vineyards (Leeb et al. 2020). In addition to ethical and logistical reasons, the use of juveniles is appropriate because they play a key role in the dispersal and thus the connectivity of populations in landscapes (Cushman 2006). We hypothesized that dermal exposure of juvenile *R. temporaria* to soil contaminated with realistic field rates of the most common German viticultural fungicide, folpet, does induce sublethal, but not lethal, effects. Moreover, we hypothesized that previous exposure in their aquatic life stages affects the sensitivity of amphibians in their subsequent terrestrial stages. Individuals that had experienced a previous aquatic pesticide exposure in the study ponds were expected to be more tolerant at their terrestrial stage because of possible adaptation processes, for example, by maintaining and improving the hepatic glutathione redox status (Peña-Llopis et al. 2003).

MATERIALS AND METHODS

Animal collection and husbandry

In March 2018, we collected parts of 4 freshly laid egg clutches (up to 24 h old) of *R. temporaria* from an uncontaminated pond in the Palatinate forest in southwest Germany (49.25475 N, 7.96182 E, WSG84). Each egg clutch was kept separately in aerated aquaria (32 × 24 × 20 cm) filled with filtered tap water (0.2 µm Supor; Pall) in a climate chamber with a 16:8-h light:dark cycle at 21 ± 1 °C. Water was renewed daily. As soon as the tadpoles reached Gosner stage 25 (Gosner 1960), they were held randomized in net cages (40 × 65 ×30 cm) in 8 ponds (ponds A–H; Table 1; Supplemental Data, S1) in the wine-growing region Südliche Weinstraße and the Palatinate forest. In total, 4 cages with 77 tadpoles were reared in each pond. Because most of the individuals were removed for samplings of preceding aquatic experiments, a limited number of juveniles was used for the present terrestrial study (Table 1). The rearing ponds represented a gradient of pesticide concentrations (Table 2; Supplemental Data, S2). As soon as the individuals metamorphosed, the juveniles were kept in outdoor cages (up to 40 individuals/cage, 40 × 65 × 30 cm) filled with forest soil, moss, leaves, and a water supply,

under natural conditions, placed outside of the research station Geilweilerhof (49.215324 N, 8.048003 E, WSG84) of the University of Koblenz-Landau. Every other day, juveniles were fed ad libitum with *Drosophila melanogaster* and *D. hydei* obtained from an in-house culture.

TABLE 1: Locations of rearing ponds and used replicate numbers of juvenile *Rana temporaria* exposed in each test with increasing pesticide contamination from A to H

		Folpan® 80 WDG		**Folpan® 500 SC**	
Pond	**Coordinates**	**Control**	**Treatment**	**Control**	**Treatment**
A	49.25475, 7.96182	8	8	-	-
B	49.23830, 7.99002	8	8	-	-
C	49.23637, 8.07850	8	8	8	8
D	49.20329, 8.20917	8	8	-	-
E	49.31700, 8.12906	8	8	7	6
F	49.21830, 8.04944	8	8	-	-
G	49.31776, 8.14445	7	6	-	-
H	49.18898, 8.03709	8	8	-	-
	Total number	63	62	15	14

SC = suspension concentrate; WDG = water dispersible granule.

TABLE 2: Number of detected pesticides, maximum sum of toxic units, frequency of Folpet detections, and determined summed Folpet concentrations for each rearing pond[a]

Pond	Pesticide frequency	SumTU	Folpet frequency	Sum folpet (µg/L)
A	0	-4.48	0	0.00
B	6	-3.48	0	0.00
C	14	-3.09	2	0.55
D	7	-2.94	0	0.00
E	14	-2.41	1	1.08
F	16	-2.25	0	0.00
G	16	-2.14	3	1.71
H	19	-1.75	4	6.04

[a]Ponds were ordered from A to H with increasing pesticide loads (sum of toxic units). Because no pesticides were detected in pond A, its sum of toxic units was calculated based on the use of one-tenth of the minimum toxic unit observed in the sites with detected concentrations.
SumTU = sum of toxic units.

Test substances

The test substances Folpan® 80 water dispersible granule (WDG) and Folpan® 500 suspension concentrate (both ADAMA Deutschland) were purchased from a local distributor. Both formulated products contain the phthalimide folpet (Chemical Abstracts Service no. 133-07-03) as active ingredient (a.i.), at concentrations of 78 to 85% (w/w) for Folpan WDG and 38 to 42% (w/w) for Folpan suspension concentrate. Folpet acts as a preventative broad-spectrum fungicide against leaf spot diseases in grapevines. It is categorized as being acutely toxic to aquatic organisms, an eye irritant, a skin sensitizer, and a suspected carcinogen. Both formulations also contain additives: Folpan WDG contains phenolsulfonic acid-formaldehyde-polycondensate as sodium salt with 2.0 to 3.0% (w/w), which is classified as chronic aquatic toxic (Adama 2016). Folpan suspension concentrate contains 3.5 to 5% (w/w) of sulfonated aromatic polymer, sodium salt, 1 to 1.5% (w/w) of fumaric acid, and 0.5 to 1% (w/w) of methenamine, the first 2 being classified as eye irritants and the third being a skin sensitizer and flammable solid (Adama 2015). Formulations were used instead of the active ingredient mainly for environmental realism but also because additives in formulations can affect amphibians (Puglis and Boone 2011; Brühl et al. 2013). Both folpet formulations can be applied

up to 8 times per growing season, with a required time interval of at least 7 d between applications.

Soil exposure tests

The 48-h soil exposure tests were performed in the laboratory with a 16:8-h light:dark cycle at 24 ± 1 °C. Prior to test initiation, all animals were acclimatized for 24 h under the same conditions. After feeding the juveniles ad libitum with *D. melanogaster* and *D. hydei*, they were weighed (±0.01 mg) and kept individually in clear, lockable plastic terrariums (22.5 × 16.5 × 7 cm; Braplast) filled with 250 g artificial soil. The soil was prepared according to Organisation for Economic Co-operation and Development guideline 207 (1984) and consisted of 70% industrial sand (particle diameter 50–200 µm; Euroquarz), 20% kaolin clay (Carl Roth), and 10% sphagnum peat (sieved through 2-mm mesh; Floratorf; Floragard).

For each of the 2 pesticide formulations, one treatment group (pesticide-exposed) and one control group (equal amount of filtered tap water) were tested with 8 individuals each. This was not possible for juveniles from each pond (Table 1; 6–8 individuals per treatment group, 154 in total) because a limited number of juveniles was available as a result of different survival rates during aquatic development. For logistical reasons, formulations could not be tested at the same time, but they were tested within 2 wk of each other. In a pretest, 3 individuals were exposed to soil contaminated with 100% of the maximum recommended field rate of Folpan WDG (FR_{max} = 3.6 kg/ha), which led to 100% mortality after 24 h. To additionally consider pesticide exposure mitigation by interception of crops (Cusaac et al. 2017), half of the maximum recommended field rate (50% FR_{max} = 1.6 kg Folpan WDG/ha) of Folpan WDG was applied to the soil, which corresponds to 0.64 kg a.i./ha with an application volume of 200 L/ha. To allow comparison of effects induced by Folpan WDG and Folpan suspension concentrate, equal amounts of the active ingredient were applied for Folpan suspension concentrate, resulting in a field rate of 1.28 L Folpan suspension concentrate/ha with an application volume of 200 L/ha. Filtered tap water was used to prepare the pesticide solution. The soil of each box was prewetted with tap water (40 mL/box) before application of the treatment solutions, to prevent dehydration. Water and pesticide solutions were applied using a laboratory spray application system (Try Spray Cabinet; Schachtner Gerätetechnik) with singular nozzles (TeeJet TP80). Within 2 h after pesticide application, the test organisms were placed in the box for 48 h. Animals were not fed during the exposure. The mortality of the individuals was assessed after 24 and 48 h of dermal exposure. After the exposure period, behavioral tests were conducted to

determine effects on locomotor activity and feeding behavior. To investigate sublethal effects on body mass, test individuals were weighed prior to test initiation and after test termination to calculate the relative body mass decline. Afterward, the juveniles were euthanized using a buffered 0.1% tricaine mesylate solution.

Beavhior analysis

To investigate sublethal effects on locomotor behavior, surviving juveniles were filmed individually for 10 min after an acclimatization period of 2 min in a circular glass arena (Petri dish with a diameter of 20 cm and height of 5 cm). Eight camera modules (suspension concentrate15-1, Kuman) connected to single-board computers (Raspberry Pi 3 Model B; Raspberry Pi Foundation) were used for filming. The video tracking software EthoVision XT (Ver 12.0; Noldus Information Technology 2017) was used to analyze the locomotor behavior, quantified as the total distance moved. To investigate effects on the predation behavior of juveniles, 5 *D. melanogaster* were offered to the treatment and control group of one pond (pond E). By filming the juveniles for 10 min, we determined the total number of flies eaten and the amount of time spent until the first catch.

Pesticide exposure and toxicity assessment

To quantify the pesticide concentration gradient in the 8 rearing ponds, 5 grab water samples from each pond were collected between April and May and analyzed externally by the Institute of Phytomedicine of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz in Neustadt/Weinstraße, Germany. The samples were analyzed for a total number of 47 different fungicides, 6 insecticides, 3 herbicides, and 2 acaricides (Supplemental Data, S2 Table 1), which were selected based on spraying recommendations from local authorities (www.dlr.rlp.de). The toxicity of the pond pesticide exposure was assessed using toxic units for each detected pesticide and sampling day (toxic unit = $C_i/LC50_i$, where C_i is the detected concentration of pesticide i and $LC50_i$ is the median lethal concentration causing 50% mortality of test organisms). The use of toxic units is more favorable compared to using mean concentrations across samples because it includes a measure of hazard toward amphibians. The combination of the concentration data and toxicity data of surrogate fish species allows a more realistic assessment of how the pond contamination could affect amphibians. The LC50 values of fish toxicity studies were used as proxy for amphibians because LC50 values for amphibians are rare. Toxicity data were compiled from the Pesticide Properties Database (Agriculture and

Environment Research Unit of the University of Hertfordshire 2013). Using the sum of toxic units is preferred over using toxic units because one toxic unit only represents the toxicity of one pesticide, and ponds were contaminated with several pesticides. To aggregate the toxicity from different pesticides, the logarithmic sum of toxic unit (n = number of detected pesticides) was calculated for each sampling day (Equation 1; for rationale, see Schäfer et al. 2011).

$$SumTU = log\left(\sum_{i=1}^{n} \frac{C_i}{LC_{50_i}}\right) \qquad (1)$$

The maximum values of the 5 sampling days were selected to define the maximum sum of toxic units because this value reflects the worst determined toxicity toward amphibians. The maximum sum of toxic units was then used to define the pesticide gradient across ponds. Sites for which no pesticides were detected were assigned a toxic unit of one-tenth of the minimum toxic unit observed in the sites with detected concentrations (leading to a sum of toxic units of –4.48 for pond A).

Statistical analyses

Statistical analyses were performed using R (Ver 3.5.2; R Development Core Team 2013). Mortality was recorded as binary data and transformed to the percentage of dead animals per group. Statistically significant differences between groups were determined using 2-sample *z* tests for equality of proportions with continuity correction (Newcombe 1998). Wilcoxon rank sum tests were used to compare control and treatment groups within ponds for numeric data (i.e., mass decline and behavioral endpoints). To analyze possible effects of the pond on the mortality, mass decline, and distance moved after the exposure to Folpan WDG, a generalized linear mixed model (GLM) was performed with “pond” as the fixed factor. To investigate possible correlations between the exposure background (sum of toxic units) and the investigated endpoints, Spearman's rank correlations were performed. The effect of sum of toxic units on the initial body mass and the influence of the latter on mortality was analyzed using Spearman's rank correlations. Moreover, the moved distance was correlated with body mass after the exposure period. For all statistical tests, the criterion for significance was set to $\alpha = 0.05$.

Animal welfare

The experiments were approved by the Federal Investigation Office (Landesuntersuchungsamt, Koblenz, Germany) to section 8a of the German law for animal welfare with the approval number 23 177-07/G18-20-009 and the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license 42/553-254/455-18).

RESULTS

Pesticide exposure and toxicity assessment

The pesticide residue analysis revealed 0 to 19 different pesticides in the rearing ponds (Supplemental Data, **S3** Table), with sum of toxic units between –4.48 and –1.75 (Table **2**). The number of folpet detections ranged from 0 to 4 sampling days with summed folpet concentrations of 0.55 to 6.04 µg/L, whereby no relation between the sum of toxic units and the sum of folpet detections (Sum folpet) can be observed.

Mortality

The 48-h exposure to both tested fungicide formulations led to mortality in *R. temporaria* juveniles (Figure 1). After 48 h, for both formulations, no control mortality was observed except for pond H (13%) of Folpan WDG. The 48-h exposure to Folpan WDG resulted in mortalities ranging from 17% (pond G) to 100% (pond H), whereby 4 treatment groups differed significantly from their respective control group (ponds B, D, F, H; $p < 0.03$; Supplemental Data, S3 Table 1). Exposure to Folpan suspension concentrate induced 13 and 17% mortality for ponds C and E, respectively. However, no significant differences between treatment and control groups could be detected ($p > 0.94$; Supplemental Data, S3 Table 1). After 24 h, 32% of the observed mortality was observed in the Folpan WDG treatment and none in the Folpan suspension concentrate treatment.

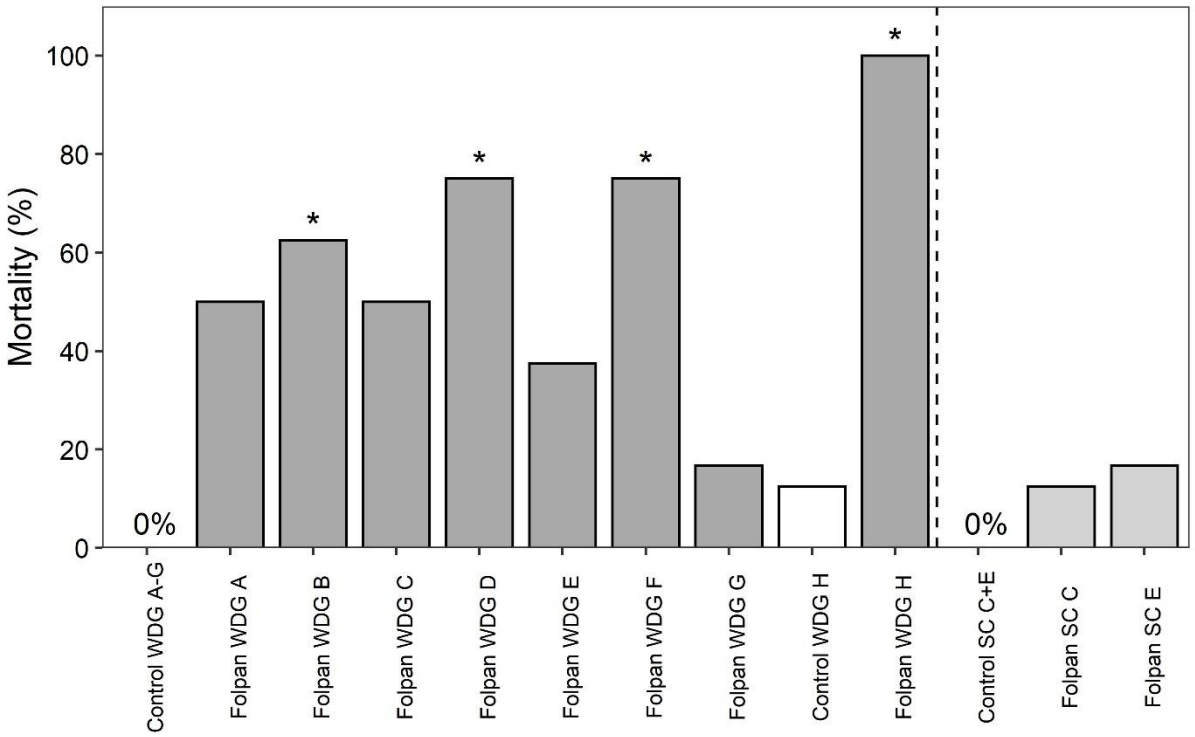

FIGURE 1: Mortality of juvenile *Rana temporaria* of each pond after 48-h soil exposure to Folpan water dispersible granule (dark gray) and suspension concentrate (light gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. *Statistically significant difference ($p < 0.05$) between control and respective treatment. SC = suspension concentrate; WDG = water dispersible granule.

Body mass decline

Dermal exposure to Folpan WDG induced a nonsignificant median body mass decline in juveniles of the fungicide treatments compared to the controls in ponds B, C, D, E, and F ranging from 0.5 (pond B) to 1.8% (pond E; Figure 2; Supplemental Data, S3 Table 2). The median mass decline in the treatment group of ponds A and G was lower than in the control (–1.95 and –0.15%), although this was not statistically significant (Supplemental Data, S3 Table 2). Folpan suspension concentrate induced a nonsignificant 3.23 and 1.75% median mass decline for ponds C and E compared to the controls (Figure 2; Supplemental Data, S3 Table 2). The GLM revealed no effect of the rearing ponds on body mass decline after exposure to Folpan WDG ($df = 6$, $p = 0.99$). No correlation could be found for the influence of the sum of toxic units on mass decline after exposure to Folpan WDG (Spearman's correlation coefficient $\rho = -0.18$, $p = 0.10$) as well as on initial body mass (Spearman's correlation coefficient $\rho = 0.16$, $p = 0.08$).

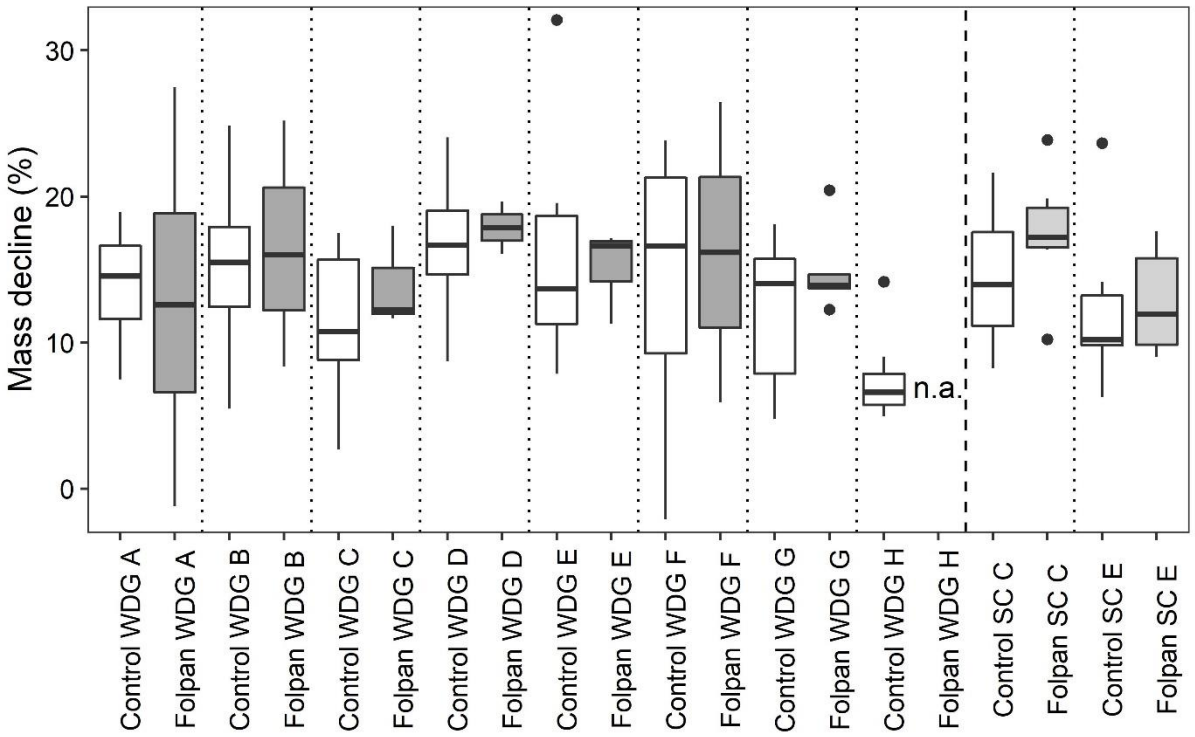

FIGURE 2: Relative mass decline of juvenile *Rana temporaria* of each pond after 48-h soil exposure to Folpan water dispersible granule (dark gray) and suspension concentrate (light gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. No statistically significant differences ($p < 0.05$) between control and respective treatment were found. The box of each boxplot represents the interquartile range given by the 25th and 75th percentiles. Whiskers correspond to the lowest and highest value within a distance of 1.5 times the 25th and 75th percentiles. Data points beyond the whiskers are shown as filled circles. n.a. = not analyzed; SC = suspension concentrate; WDG = water dispersible granule.

Total distance moved

Folpan WDG induced a median decreased locomotor activity in every pond (Figure 3), ranging from 8.86% (pond E) to 72.64% (pond F). Wilcoxon rank sum tests resulted in a significant difference only for the treatment group to the control of pond G (65.57%, $p = 0.048$; Supplemental Data, S3 Table 3). Exposure to Folpan suspension concentrate resulted in a nonsignificant 85.15% reduction and a 31.65% increase of the median total distance moved for C and E, respectively (Figure 3; Supplemental Data, S3 Table 3). The GLM revealed no effect of the rearing ponds on the total distance moved after exposure to Folpan WDG ($df = 6$, $p = 0.21$). No correlation could be found for the influence of sum of toxic units on total distance moved after exposure to Folpan WDG (Spearman's correlation coefficient

$\rho = 0.04$, $p = 0.71$). Moreover, body mass after 48 h did not affect the moved distance (Spearman's correlation coefficient $\rho = 0.10$, $p = 0.36$).

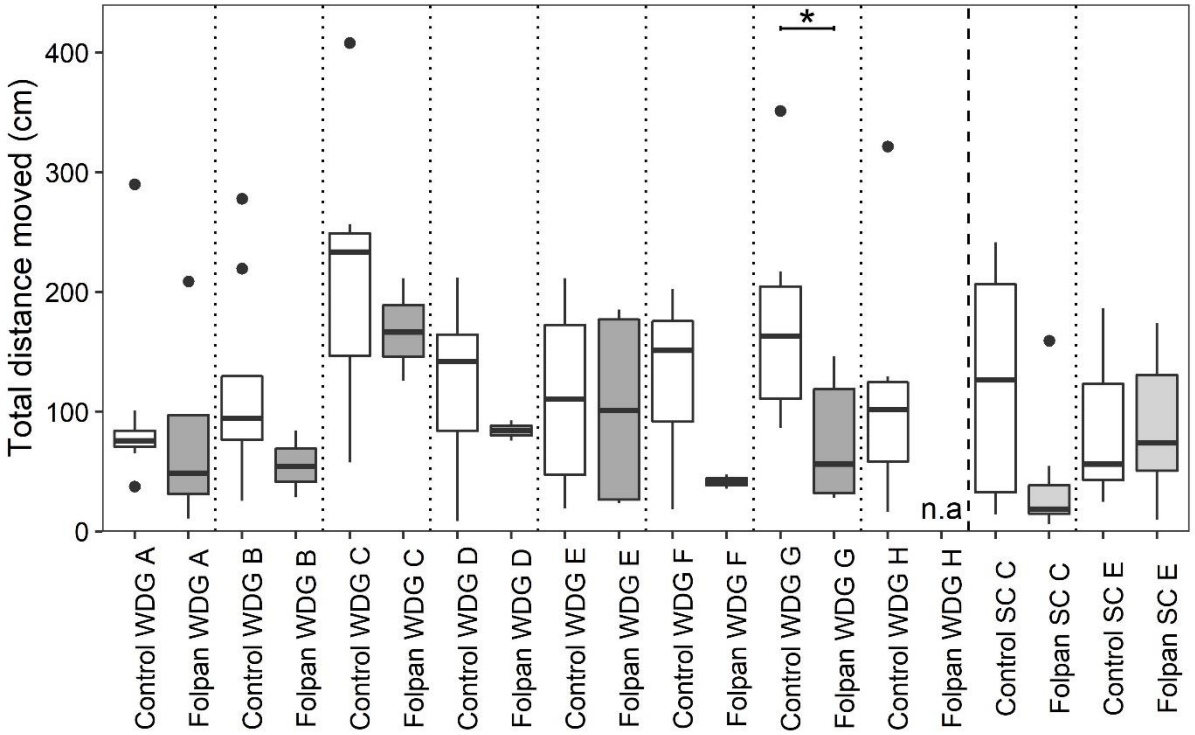

FIGURE 3: Total distance moved of juvenile *Rana temporaria* of each pond after 48-h soil exposure to Folpan water dispersible granule (dark gray) and suspension concentrate (light gray). Letters increasing from A to H represent the aquatic pesticide background from no to high maximum sum of toxic units. The box of each boxplot represents the interquartile range given by the 25th and 75th percentiles. Whiskers correspond to the lowest and highest value within a distance of 1.5 times the 25th and 75th percentiles. Data points beyond the whiskers are shown as filled circles. *Statistically significant difference ($p < 0.05$) between control and respective treatment. n.a. = not analyzed; SC = suspension concentrate; WDG = water dispersible granule.

Feeding behavior

The feeding behavior test revealed a nonsignificant mean reduction of 20% of consumed *D. melanogaster* after 48-h exposure to Folpan WDG ($p = 0.11$) and a significant reduction of 47% after 48-h exposure to Folpan suspension concentrate ($p = 0.01$; Figure 4; Supplemental Data, S3 Table 4). Moreover, for both fungicides nonsignificant mean increases of 50 and 169% in necessary time to catch the first *D. melanogaster* could be observed (Figure 5; Supplemental Data, S3 Table 5; Folpan WDG $p = 0.51$, Folpan suspension concentrate $p = 0.49$).

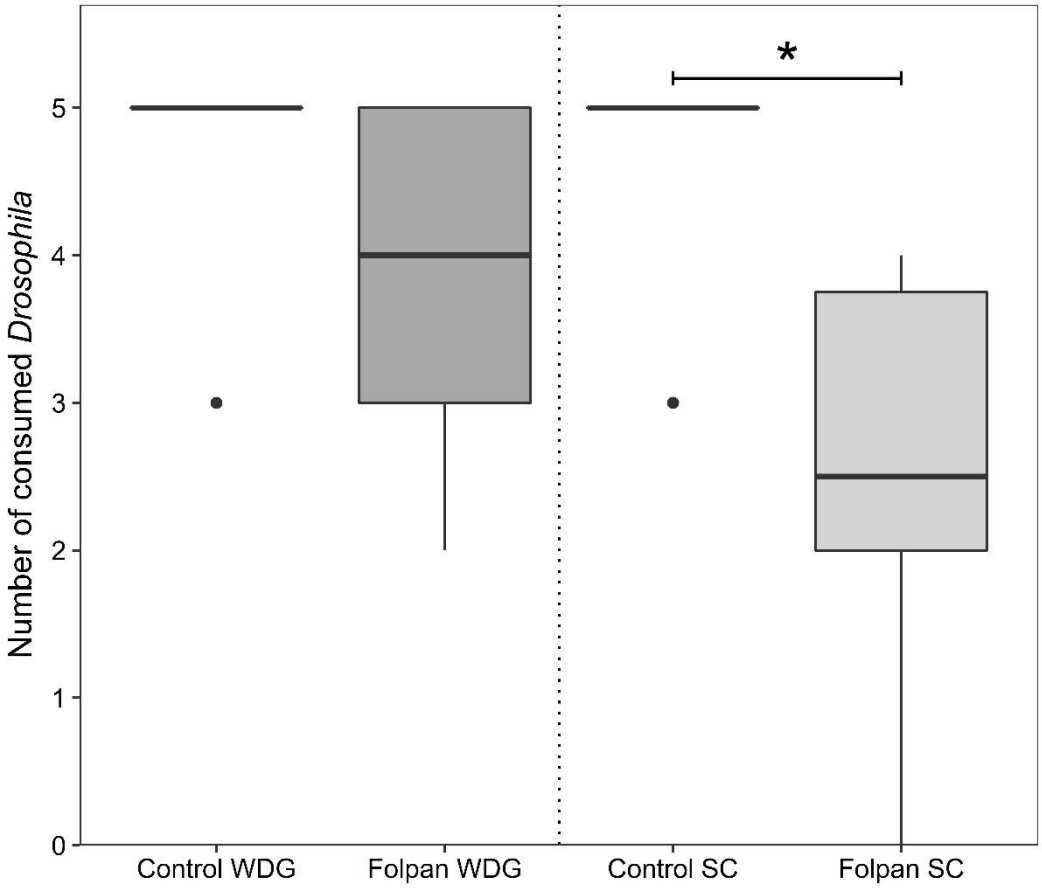

FIGURE 4: Number of consumed *Drosophila melanogaster* by juvenile *Rana temporaria* in control and treatment groups of pond E after 48-h soil exposure to Folpan water dispersible granule (dark gray; control $n = 8$, treatment $n = 5$) and suspension concentrate (light gray, control $n = 7$, treatment $n = 6$). The box of each boxplot represents the interquartile range given by the 25th and 75th percentiles. Whiskers correspond to the lowest and highest value within a distance of 1.5 times the 25th and 75th percentiles. Data points beyond the whiskers are shown as filled circles. *Statistically significant difference ($p < 0.05$) between control and respective treatment. SC = suspension concentrate; WDG = water dispersible granule.

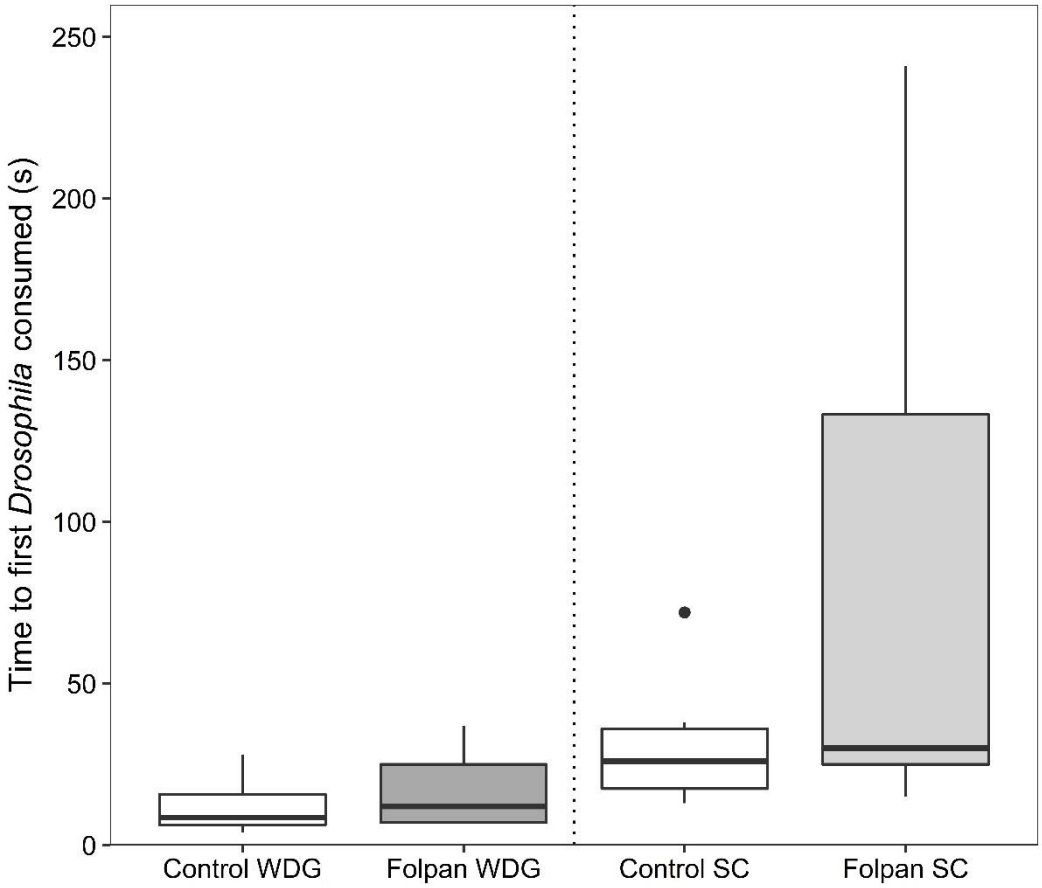

FIGURE 5: Time that juvenile *Rana temporaria* needed to catch the first *Drosophila melanogaster* in control and treatment groups of pond E after 48-h soil exposure to Folpan water dispersible granule (dark gray, control $n = 8$, treatment $n = 5$) and suspension concentrate (light gray, control $n = 7$, treatment $n = 6$). The box of each boxplot represents the interquartile range given by the 25th and 75th percentiles. Whiskers correspond to the lowest and highest value within a distance of 1.5 times the 25th and 75th percentiles. Data points beyond the whiskers are shown as filled circles. *Statistically significant difference ($p < 0.05$) between control and respective treatment. SC = suspension concentrate; WDG = water dispersible granule.

DISCUSSION

The application of pesticides in agricultural landscapes can result in mortality among nontarget organisms including amphibians. Although a scientific opinion on the state of the science on pesticide risk assessment for amphibians was formulated by the European Food Safety Authority (Ockleford et al. 2018), amphibians are not yet considered in the environmental risk assessment of pesticides. Although postmetamorphic fitness is proven to have a strong influence on amphibian population dynamics (Biek et al. 2002), aquatic life stages of

amphibians have received more attention than terrestrial life stages in ecotoxicological testing and validation of risk-assessment approaches. Because data involving terrestrial life stages of amphibians are considerably rare, the results of the present study contribute to the understanding of amphibian conservation in agricultural landscapes.

Impact of the aquatic exposure background

No correlation could be determined for ecotoxicological responses and aquatic pesticide exposure background (sum of toxic units). We hypothesized that animals originating from contaminated ponds react less sensitively to terrestrial fungicide exposure because of an adaptation to pesticides during the aquatic phase (Peña-Llopis et al. 2003; Cothran et al. 2013). However, water bodies in agricultural landscapes are often contaminated with not only one pesticide but mixtures of pesticides, exhibiting diverse modes of action and toxicity profiles. Understanding these multiple stressors is difficult because their combined effects cannot be predicted by single-stressor studies. Thus, adaptation to one or a few pesticides or survival after exposure to these pesticides does not necessarily result in tolerance to other pesticides. No evidence was found that the sensitivity response of amphibians to folpet varies with a previous aquatic pesticide exposure background. However, because of the fast degradability (50% degradation time [DT50] < 0.05 d) of folpet in water (Adama 2015) and the limited number of water samplings ($n = 5$), it is possible that in reality folpet occurs in higher and more frequent peak concentrations than detected in the residual analysis. Thus, no general conclusion can be drawn about the relation between the aquatic pesticide exposure and the terrestrial sensitivity of the juveniles to folpet. However, it should be noted that aquatic pesticide exposure did not result in weakened animals and therefore did not adversely affect the response of terrestrial juveniles either.

Mortality

Our study design did not assess the effects of an overspray scenario as did Brühl et al. (2013) but did assess dermal uptake of contaminated soil. It is remarkable that this exposure pathway at only half of the recommended FR_{max} of the most common viticultural fungicide in Germany results in such high mortality levels in juvenile *R. temporaria*. Although not statistically significant, also the detected mortalities of 17 to 50% after 48 h (Figure 1) need to be considered as ecologically relevant. Because of these mortality rates, adverse effects on populations seem likely, which might have contributed to the population declines, especially on a local level.

No mortality data for folpet involving postmetamorphic amphibians could be retrieved in a literature review. However, high mortality was observed in amphibians following terrestrial exposure to another phthalimide fungicide, captan (Brühl et al. 2013). Brühl et al. (2013) recorded mortalities of 100% of juvenile *R. temporaria* 7 d after direct overspray with a captan formulation at 100% of the recommended FR_{max} as well as 40% mortality at already 10% FR_{max}, which shows that captan—and likely its sister compound folpet—is harmful to amphibians. Other studies report mortality rates of 0 to 100% after exposure to various pesticide formulations through direct overspray and contaminated soil, suggesting that toxicity varies by pesticide class (Bernal et al. 2009; Storrs Méndez et al. 2009; Belden et al. 2010; van Meter et al. 2014; Cusaac et al. 2015).

These studies and the results of the present study emphasize the necessity to include terrestrial life stages in the environmental risk assessment of pesticides.

Sublethal effects

Dermal exposure to the 3 fungicide formulations induced a nonsignificant overall body mass decline compared to the control. This is in line with another study, in which neither terrestrial nor aquatic exposure to the insecticide carbaryl appeared to be detrimental to the growth of juvenile Great Plains toads (*Anaxyrus cognatus*) after 42 d (Webber et al. 2010). However, with respect to the great importance of water for amphibians, the small reduction in body mass detected in the present study could represent a water loss which may already indicate stress regarding the osmoregulation affecting vital functions of the animals (Shoemaker and Nagy 1977).

Because of the juveniles' key role in the dispersal of amphibians (Cushman 2006), the reduced activity after dermal exposure to contaminated soil could further contribute to local amphibian declines. Moreover, reduced activity could also lead to adverse predator–prey behavior as well as an increased vulnerability to dehydration because juveniles may need longer to reach water supplies. In addition, the results show that dermal exposure via soil affects the feeding behavior. The presented results may even underestimate this effect because we offered only a limited number of flies to prey on, and the control group might have eaten more flies than offered. Bracher and Bider (1982) found similar effects on the feeding activity of an American toad (*Bufo americanus*) forest community after a terrestrial carbamate exposure. Likewise, Mitchkash et al. (2014) recorded effects on the best run time of spotted salamander (*Ambystoma*

maculatum) after carbaryl exposure. In contrast, Webber et al. (2010) and Cusaac et al. (2017) did not find any effects on feeding behavior after exposure of juvenile Great Plains toads (*Anaxyrus cognatus*) to carbaryl and pyraclostrobin.

The differing results for the presented sublethal effects like mass decline and behavior changes compared to these studies may be due to different species sensitivities (Bridges and Semlitsch 2000), different modes of action of the pesticides, different spray scenarios, and the use of various underground substrates like wet towels or soil compositions. This highlights the need for standardized protocols for postmetamorphic, terrestrial amphibian testing to allow a more precise comparison among studies and pesticides.

Because of the high mortality rates in several treatments in the present study, especially for Folpan WDG, the number of individuals for which sublethal effects could be observed and analyzed was reduced. Compared to other ponds, for which mortalities ranged from 37.5 to 100%, only 17% of individuals from pond G died. Therefore, the number of replicates for sublethal observations, and thus the statistical power, was higher compared to other treatments. This may have led to the statistically significant decrease in the total moved distance in pond G. Thus, also nonsignificant results of the investigated sublethal endpoints need to be considered carefully.

Formulation effects

In both folpet tests, the same amount of active ingredient was applied to the soil. Thus, the faster and significantly higher mortality after the exposure to Folpan WDG compared to Folpan suspension concentrate indicates that the formulation-specific toxicity must be due to the differing additives. Folpan suspension concentrate was expected to be more toxic because of the properties of its additives, which are classified as eye-irritating and skin-sensitizing in contrast to the additive of Folpan WDG, which is classified as chronically toxic to aquatic organisms. Folpan WDG has a 5-fold lower 48-h LC50 for *Daphnia magna* than Folpan suspension concentrate (0.68 vs 3.9 mg/L, respectively (Adama 2015, 2016), indicating that Folpan WDG may be more toxic than Folpan suspension concentrate nonetheless. However, the varying toxicity may also be induced because of the additives altering the absorption and metabolism properties of the formulation and not because of their own toxicity.

The present results underline the urgent need to involve not only active ingredients but also the additives in the environmental risk assessment of pesticides to nontarget organisms, including

amphibians. However, it is difficult to understand the role of additives because they are indicated as proprietary information in many formulations and the exact chemical composition is not declared, therefore complicating a comprehensive evaluation of the toxicity of pesticide formulations.

Potential for mitigation and environmental relevance

The overlap between the application of pesticides and the presence of amphibians in agricultural landscapes plays an important role in the assessment of the environmental relevance. Although the present study was performed under controlled laboratory settings with a direct overspray of the soil without any interception of crops, which decreases the amount of pesticides reaching the soil, the use of only 50% of the maximum recommended field rate is considered to represent a worst-case realistic exposure scenario and is thus also environmentally relevant. Furthermore, in Germany amphibian mating usually takes place in March and April (Günther 2009). In the investigated viticultural study area, regular folpet applications start at the end of May (Leeb et al. 2020). Metamorphs used in the present study were leaving the more contaminated ponds (ponds E–H) in the first week of June, indicating a high risk of being exposed to soil contaminated with folpet. Certainly, the burying behavior and the mainly nocturnal movement of amphibians (Günther 2009) may decrease the risk of pesticide exposure during and after their application; however, considering the DT50 of folpet of 4.3 d in soil, this still indicates a threat to amphibians moving through vineyards in the first few days after application.

In a telemetry study from Leeb et al. (2020), it has been observed that adult common toads, *Bufo bufo*, can migrate through the viticultural landscape to their winter habitats in adjacent forests. Although vineyards were used 23% less often as a habitat than expected from a random habitat choice, Leeb et al. found toads staying in vineyards over a longer time period (on average 123 d), suggesting that a part of the population inhabits the agricultural landscape during large parts of the year. Moreover, they determined that >75% of the population may be directly exposed to a pesticide application and that up to 24% of the population can temporarily come into contact with pesticides within a day. Recapitulating these circumstances, the present study can be considered a realistic worst-case exposure field scenario.

CONCLUSION

The present study found that dermal exposure of amphibians to soil contaminated with folpet formulations can lead to lethal and sublethal effects, which thereby increases the understanding of how folpet affects postmetamorphic, terrestrial amphibian life stages. In the presented worst-case exposure scenario, 0 to 83% of the progeny would survive exposure to soil contaminated with folpet. The surviving juveniles might additionally suffer from sublethal effects like impairments of activity and predation behavior, which could have both immediate and long-term fitness effects. In conclusion, our results show that exposure to the most common German fungicide may contribute to the severe decreases of amphibian populations in viticultural landscapes of Germany. Given that folpet causes such lethal and sublethal effects despite its rapid degradation in soil, the question arises whether other, more stable pesticides with similar toxicities might pose an even higher threat to amphibians.

Our laboratory setup represents a lower-tier study with a small replicate number for the investigated sublethal effects because of the previous high mortality rates. Therefore, we recommend that additional higher-tier studies be performed to reflect more realistic exposure scenarios which include the presence of additional stressors. Furthermore, a comprehensive toxicity evaluation of fungicides to amphibians and the reduction of applications of highly toxic products are needed to maintain long-term survival of amphibian populations in agricultural landscapes.

Supplemental Data

The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4972.

Acknowledgment

We thank C. Leeb for assistance during the experiment, generation of the study sites map, and valuable comments on earlier drafts of this paper. Thanks to C. Hoffmann, T. Gramm, and N. Steinmetz of the Julius Kühn-Institut for allowing us to use the laboratory spray application system and the help during application processes. We thank V. Sesin for her language check and valuable comments on this paper. We are also grateful to M. Entling for providing access to the software Ethovision. Sincere thanks to A. Kortekamp and M. Twertek of the

Dienstleistungszentrum Ländlicher Raum Rheinpfalz for the pesticide residue analysis. Further, we thank S. Kolbenschlag for assistance during the experiment as well as S. Andermann, L. Bleidt, M. Müller, and R. Pleitner for assistance during the aquatic rearing phase of tadpoles. Open Access funding enabled and organized by ProjektDEAL.

Disclaimer

The authors declare that funding for the present study was provided by the PhD scholarship program of the German Federal Environmental Foundation.

Conflict of interest

The authors declare that there is no conflict of interest.

Author Contribution Statement

E. Adams, C.A. Brühl, V. Gerstle: study conception and design; E. Adams, V. Gerstle: laboratory experiments, data analysis, writing–draft. All authors contributed to and approved the final manuscript.

Data Availability Statement

Data, associated metadata, and calculation tools are available from the corresponding author (adams@uni-landau.de).

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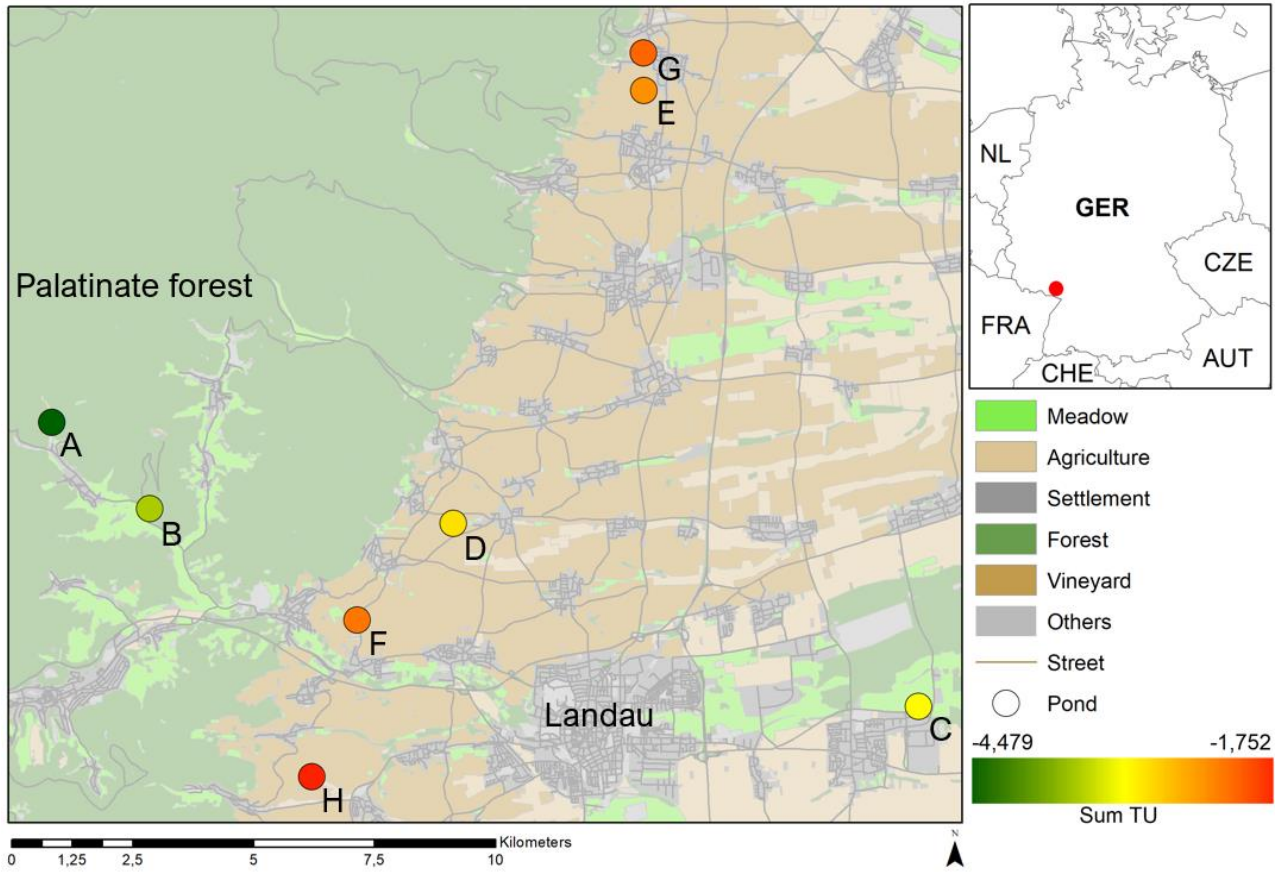
Supporting information of scientific publication 3

S 1. Information about the rearing ponds.



S 1 Figure 1. Map of study area with rearing ponds, in which the aquatic development took place.

Source: Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data]

S 1 Table 1. Measured parameters during water samplings. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 (rain event), 24.05.2018). pH values, oxygen concentration, temperature and conductivity were measured using a WTW multiparameter MultiLine Multi 340i and a WTW SenTix pH-electrode with a temperature sensor SenTix 41, a WTW oxygen sensor CellOx 325 and a WTW conductivity electrode TetraCon 325 (WTW, Weilheim in Oberbayern, Germany). Measuring kits of JBL GmbH & Co. KG (Neuhofen, Germany) were used to measure ammonium, silicate, phosphate and nitrite.

	Pond A	Pond B	Pond C	Pond D	Pond E	Pond F	Pond G	Pond H
Sampling 1								
pH	7.98	8.3	7.98	7.28	7.73	7.91	9.29	8.11
O_2 (mg/L)	41.96	13.94	11.23	11.8	10.6	17.6	15.7	14.5
Temperature (°C)	6.2	9.6	15.6	10.0	13.1	9.9	15.9	10.7
Conductivity (yS/cm)	83	447	179	203	368	885	320	761
Ammonium (mg/L)	0.1	0.1	0.2	<0.05	<0.05	<0.05	0.2	0.1
Silicate (mg/L)	3	6	1.2	6	1.2	2	6	6
Nitrate (mg/L)	1	1	1	1	1	5	1	1
Phosphate (mg/L)	<0.02	<0.02	<0.02	<0.02	<0.6	<0.02	>1.8	<0.02
Nitrite (mg/L)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sampling 2								
pH	8.64	8.51	7.77	7.64	7.64	8.00	7.29	7.7
O_2 (mg/L)	10.53	12.6	9.95	9.74	5.92	13.98	6.69	7.06
Temperature (°C)	17.7	17.3	25.2	12.7	16.5	18.6	18.4	16.1
Conductivity (yS/cm)	76	733	184	205	328	834	277	727

Ammonium (mg/L)	<0.05	<0.05	<0.05	0.1	0.1	0.1	0.2	0.1
Silicate (mg/L)	6	6	0.4	6	0.4	6	6	4
Nitrate (mg/L)	1	1	01	1	<0.5	1	0.5	<0.5
Phosphate (mg/L)	<0.02	<0.02	<0.02	0.05	0.6	<0.02	>1.8	0.05
Nitrite (mg/L)	<0.01	<0.01	<0.01	0.025	<0.01	0.05	<0.01	<0.01
Sampling 3								
pH	7.07	8.81	7.56	7.2	7.47	7.83	7.48	7.73
O_2 (mg/L)	11.96	13.25	9.25	8.96	4.7	5.21	5.85	3.35
Temperature (°C)	10.7	14.1	17.9	10.1	13.4	16.2	13.8	14.8
Conductivity (yS/cm)	78	442	220	188	343	906	296	879
Ammonium (mg/L)	<0.05	0.1	<0.05	0.01	0.1	0.2	0.1	<0.05
Silicate (mg/L)	6	4	2	6	0.8	4	3	3
Nitrate (mg/L)	<0.5	1	1	1	<0.5	1	0.5	<0.5
Phosphate (mg/L)	<0.02	<0.02	<0.02	0.05	0.8	<0.02	>1.8	<0.02
Nitrite (mg/L)	<0.01	<0.01	<0.01	0.025	<0.01	0.1	<0.01	<0.01
Sampling 4								
pH	7.27	7.12	7.56	6.67	7.41	7.63	7.18	7.62
O_2 (mg/L)	13.5	14.4	6.4	8.31	7.88	3.28	12.65	1.99
Temperature (°C)	11.8	16.1	20.3	13.8	14.1	16.9	15.8	16.8
Conductivity (yS/cm)	78	437	228	178	347	915	270	916
Ammonium (mg/L)	<0.05	0.05	<0.05	0.1	<0.05	0.6	<0.05	0.4

Silicate (mg/L)	6	6	3	6	2	6	6	6
Nitrate (mg/L)	<0.01	1	1	<0.5	0.025	10	1	<0.5
Phosphate (mg/L)	<0.02	<0.02	<0.02	<0.02	1.8	0.2	>1.8	0.05
Nitrite (mg/L)	<0.01	0.025	<0.01	0.025	<0.5	0.2	1	0.025
Sampling 5								
pH	8.45	8.56	7.15	7.13	7.04	7.69	7.01	7.72
O_2 (mg/L)	11.3	11.37	8.07	8.07	3.48	5.51	3.61	5.48
Temperature (°C)	12.0	17.3	25.9	14.5	18.3	21.1	18.4	18.9
Conductivity (yS/cm)	83	426	210	197	290	854	193	813
Ammonium (mg/L)	<0.05	<0.05	<0.05	0.2	<0.05	0.3	<0.05	0.1
Silicate (mg/L)	6	6	3	6	3	6	3	6
Nitrate (mg/L)	<0.5	<0.5	0.5	1	<0.5	5	<0.5	<0.5
Phosphate (mg/L)	<0.02	0.05	<0.02	0.4	1.8	0.05	1.2	<0.02
Nitrite (mg/L)	0.025	0.05	<0.01	0.05	<0.01	0.15	0.025	<0.01

S 1 Table 2. Relative water-level development during water samplings. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 (during rain event), 24.05.2018). The water-level change is given in a relative way in cm to water-level of sampling before.

Sampling	Pond A	Pond B	Pond C	Pond D	Pond E	Pond F	Pond G	Pond H
2	+ 2	± 0	+ 3	+ 1	+ 2	± 0	± 0	+ 3
3	+ 2	+ 7	- 3	+ 5	+ 14	+ 10	+ 4	+ 12
4	- 2	± 0	+ 3	- 8	+ 2	+ 8	+ 2	- 12
5	+ 10	+ 5	- 11	+ 10	+ 1	+ 1	- 2	- 1

S 1 Table 3. Water body characteristics: Type, relative pond depth, water level permanence, size, relative vegetation level at shore inflow, drain.

Pond	Water body type	Relative pond depth	Water level	Size (ha)	Relative vegetation level at shore	Inflow?	Drain?
A	Artificial pond	Shallow	Permanent	0.011	High	Yes	Yes
B	Last pond of a pond system with three subsequent ponds	Deep	Permanent	0.344	Low	Yes	Yes
C	Natural water accumulation between inflow and drain	Shallow	Permanent	0.071	Low	Yes	Yes
D	Natural pond	Shallow	Permanent	0.192	Medium	No	No
E	Natural pond	Shallow	Non-Permanent	0.065	High	No	No
F	Artificial fish pond	Deep	Permanent	0.109	Medium	Yes	Yes
G	Artificial rain retention pond	Shallow	Permanent	0.106	High	No	No
H	Artificial rain retention pond	Deep	Permanent	0.206	Low	Yes	Yes

S1 Table 4. Surrounding environment of study ponds in a radius of 1000 m. Based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50) the percentage of vineyards, other agriculture (without meadows), meadows, settlements and forests was calculated.

Pond	Vineyard (%)	Other Agriculture (%)	Meadow (%)	Settlement (%)	Forest (%)	Other (%)
A	0.00	0.00	3.21	2.51	91.28	2.99
B	0.00	0.00	26.89	0.76	72.27	0.08
C	0.00	7.15	43.01	29.66	18.23	1.96
D	84.24	3.64	1.56	8.59	0.42	1.56
E	78.61	0.12	7.11	8.57	3.48	2.12
F	82.60	0.00	0.22	15.12	0.83	1.23
G	71.70	0.73	0.32	23.06	3.82	0.38
H	85.56	8.09	1.41	3.95	0.47	0.52

S 2. Information about the pesticide analyses in ponds.

S2 Table 1. Investigated target pesticides of the aquatic residual analysis. In total 47 different fungicides, six insecticides, three herbicides and two acaricides were investigated.

Fungicides	Insecticides	Herbicides	Acaricides
Amisulbrom	Chlorpyrifos-methyl	Atrazine	Spirodiclofen
Azoxystrobin	Dimethoate	Carfentrazone-ethyl	Tebufenpyrad
Benalaxyl-M	Indoxacarb	Simazine	
Benthivalicarb	Methidathion		
Boscalid	Parathion-ethyl		
Captan	Parathion-methyl		
Cyazofamid			
Cyflufenamid			
Cyprodinil			
Dichlofluanid			
Difenoconazole			
Dimethomorph			
Epoxiconazole			
Famoxadone			
Fenarimol			
Fenhexamid			
Fenpropimorph			
Fenpyrazamine			
Folpet			
Fludioxonil			
Fluopicolide			
Fluopyram			
Fluquinconazol			
Iprodion			
Iprovalicarb			
Kresoxim-methyl			
Metalaxyl M			
Metrafenone			
Myclobutanil			

Penconazole			
Prochloraz			
Procymidon			
Propinconazole			
Proquinazid			
Pyraclostrobin			
Pyrifenox			
Pyrimethanil			
Quinoxyfen			
Spiroxamin			
Tebuconazole			
Tetraconazole			
Tolylfluanid			
Triadimefon			
Triadimenol			
Trifloxystrobin			
Vinclozolin			
Zoxamide			

S2 Table 2. Detected pesticide concentrations in the aquatic residual analysis. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 (rain event), 24.05.2018). Folpet detections are highlighted in bold.

	Pond A		Pond B		Pond C		Pond D	
	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)
Sampling 1	-	-	Boscalid Fludioxonil Dimethoate	0.09 0.04 0.03	Azoxystrobin Difenconazole Fludioxonil Iprovalicarb Metalaxyl M Myclobutanil Dimethoate	0.04 0.02 0.07 0.28 0.08 0.03 0.05	Cyprodinil Tebuconazole Dimethoate	0.03 0.06 0.04
Sampling 2	-	-	Boscalid Zoxamide	0.02 0.03	Azoxystrobin Fluopyram Zoxamide	0.03 0.02 0.12	Zoxamide	0.04
Sampling 3	-	-	Dimethoate	0.03	**Folpet** Iprovalicarb	0.33 0.09	-	-
Sampling 4	-	-	Fludioxonil Myclobutanil Dimethoate	0.06 0.14 0.02	Azoxystrobin Fludioxonil Iprovalicarb Myclobutanil	0.02 0.05 0.11 0.04	Cyprodinil Fludioxonil Myclobutanil Pyrimethanil	0.05 0.24 0.06 0.02

					Pyrimethanil Dimethoate	0.02 0.03	Tebuconazole Dimethoate	0.21 0.38
Sampling 5	-	-	Boscalid Fludioxonil Penconazole	0.02 0.07 0.02	Cyflufenamid **Folpet** Fludioxonil Iprovalicarb Metrafenon Myclobutanil Tebuconazole Chlorpyrifos-methyl Dimethoate	0.02 0.22 0.07 0.11 0.10 0.10 0.07 0.02 0.04	Tebuconazole Dimethoate	0.02 0.04

S2 Table 1 Continued. Detected pesticide concentrations in the aquatic residual analysis. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 (rain event), 24.05.2018). Folpet detections are highlighted in bold.

	Pond E		Pond F		Pond G		Pond H	
	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)	Pesticide	Conc. (µg/L)
Sampling 1	Azoxystrobin	0.04	Dimethomorph	0.06	Azoxystrobin	0.02	Azoxystrobin	0.07
	Boscalid	0.32	Famoxadone	0.06	Fludioxonil	0.06	Boscalid	0.27
	Fluopyram	0.25	Iprovalicarb	0.44	Fluopyram	0.32	Fludioxonil	0.07
	Myclobutanil	0.07	Metalaxyl M	0.08	Iprovalicarb	0.23	Fluopicolide	0.04
	Tebuconazole	0.06	Myclobutanil	0.22	Kresoxim-methyl	0.15	Fluopyram	0.21
	Trifloxystrobin	0.08	Dimethoate	0.03	Myclobutanil	0.05	Iprovalicarb	0.12
	Dimethoate	0.04			Tebuconazole	0.02	Metrafenon	0.21
					Dimethoate	0.09	Tetraconazole	0.02
Sampling 2	Boscalid	0.07	Boscalid	0.04	Boscalid	0.24	Boscalid	0.02
	Fluopyram	0.17	Fluopicolide	0.04	Dimethomorph	0.06	Dimethomorph	0.1
	Iprovalicarb	0.46	Zoxamide	0.04	**Folpet**	0.79	**Folpet**	4.53
	Myclobutanil	0.08			Fluopicolide	0.12	Fluopicolide	0.04
					Fluopyram	0.22	Fluopyram	0.13
					Kresoxim-methyl	0.28	Iprovalicarb	3.05
					Metrafenon	0.08	Kresoxim-methyl	0.22
					Myclobutanil	0.05	Metrafenon	0.05

					Penconazole	0.04	Myclobutanil	0.73
					Zoxamide	0.41	Penconazole	0.03
							Pyrimethanil	0.03
							Zoxamide	0.14
							Dimethoate	0.06
Sampling	Boscalid	0.22	Dimethoate	0.03	Boscalid	0.17	**Folpet**	0.58
3	Fluopyram	0.34			Fludioxonil	0.24	Fludioxonil	0.07
	Myclobutanil	0.06			Myclobutanil	0.04	Fluopyram	0.20
	Tebuconazole	0.09			Tebuconazole	0.07	Dimethoate	0.02
	Zoxamide	0.04						
Sampling	Azoxystrobin	0.05	Boscalid	0.02	Azoxystrobin	0.04	Boscalid	0.15
4	Boscalid	0.28	Cyflufenamid	0.02	Boscalid	0.29	**Folpet**	0.20
	Fluopicolide	0.24	Fludioxonil	0.19	**Folpet**	0.48	Fludioxonil	0.22
	Fluopyram	0.35	Fluopyram	0.17	Fludioxonil	0.41	Fluopyram	0.18
	Iprovalicarb	0.05	Iprovalicarb	0.12	Fluopyram	0.43	Iprovalicarb	0.11
	Metrafenon	0.12	Myclobutanil	0.06	Metrafenon	0.07	Myclobutanil	0.03
	Myclobutanil	0.07	Penconazole	0.18	Myclobutanil	0.11	Chlorpyrifos-	0.02
	Tebuconazole	0.15	Pyrimethanil	0.04	Tebuconazole	0.08	methyl	0.03
	Trifloxystrobin	0.03	Tebuconazole	0.08	Zoxamide	0.02	Dimethoate	
	Zoxamide	0.12	Chlorpyrifos-	0.02				
	Dimethoate	0.02	methyl	0.08				

			Dimethoate					
Sampling 5	Azoxystrobin	0.02	Famoxadone	0.05	Azoxystrobin	0.02	Boscalid	0.26
	Boscalid	0.42	Fludioxonil	0.06	Boscalid	0.09	Famoxadone	0.15
	Cyflufenamid	0.05	Iprovalicarb	0.11	Dimethomorph	0.07	**Folpet**	0.73
	Folpet	1.083	Myclobutanil	0.03	Famoxadone	0.06	Fludioxonil	0.25
	Fluopicolide	0.15	Dimethoate	0.03	**Folpet**	0.44	Fluopicolide	0.53
	Fluopyram	0.44			Fludioxonil	0.22	Fluopyram	0.23
	Kresoxim-methyl	0.07			Fluopyram	0.24	Iprovalicarb	0.36
	Myclobutanil	0.1			Iprovalicarb	0.19	Metalaxyl M	0.24
	Tebuconazole	0.18			Penconazole	0.04	Metrafenon	0.3
	Zoxamide	0.09			Tebuconazole	0.09	Myclobutanil	0.24
	Dimethoate	0.06					Tebuconazole	0.05
							Zoxamide	0.02
							Chlorpyrifos-methyl	0.02

S 3. Results of statistical analyses.

S3 Table 1. Test statistics of the 2-sample test for equality of proportions with continuity correction which was used to compare control and treatment mortalities within ponds.

	p-value		X^2		df		95% lower and upper confidence interval			
	Folpan WDG	Folpan SC	Folpan WDG	Folpan SC	Folpan WDG	Folpan SC	Folpan WDG		Folpan SC	
							LCI	UCI	LCI	UCI
Pond A	0.083	-	3.00	-	1	-	-0.97	-0.03	-	-
Pond B	0.031	-	4.65	-	1	-	-1.00	-0.16	-	-
Pond C	0.083	1.000	3.00	0.00	1	1	-0.97	-0.03	-0.48	0.23
Pond D	0.010	-	6.67	-	1	-	-1.00	-0.32	-	-
Pond E	0.200	0.936	1.64	0.01	1	1	-0.84	0.09	-0.62	0.29
Pond F	0.010	-	6.67	-	1	-	-1.00	-0.32	-	-
Pond G	0.936	-	0.01	-	1	-	-0.62	0.29	-	-
Pond H	0.003	-	9.14	-	1	-	-1.00	-0.52	-	-

S3 Table 2. Test statistics of the Wilcoxon rank sum test which was used to compare control and treatment mass declines within ponds. “n.a.” is given for numeric Pond H results because of a 100% mortality in the treatment group.

	p-value		Wilcoxon test statistic W	
	Folpan WDG	Folpan SC	Folpan WDG	Folpan SC
Pond A	0.933	-	17	-
Pond B	0.921	-	11	-
Pond C	0.279	0.281	6	19
Pond D	0.711	-	6	-
Pond E	0.943	0.836	19	18
Pond F	0.711	-	6	-
Pond G	0.755	-	15	-
Pond H	n.a.	-	n.a.	-

S3 Table 3. Test statistics of the Wilcoxon rank sum test which was used to compare control and treatment total distances moved within ponds. “n.a.” is given for numeric Pond H results because of a 100% mortality in the treatment group.

	p-value		Wilcoxon test statistic W	
	Folpan WDG	Folpan SC	Folpan WDG	Folpan SC
Pond A	0.283	-	23	-
Pond B	0.194	-	19	-
Pond C	0.376	0.054	17	45
Pond D	0.400	-	12	-
Pond E	0.808	0.836	18	23
Pond F	0.400	-	12	-
Pond G	0.048	-	30	-
Pond H	n.a.	-	n.a.	-

S3 Table 4. Test statistics of the Wilcoxon rank sum test which was used to compare control and treatment number of *Drosophila melanogaster* consumed of pond E individuals.

p-value		Wilcoxon test statistic W	
Folpan WDG	Folpan SC	Folpan WDG	Folpan SC
0.007	0.107	29.5	39.5

S3 Table 5. Test statistics of the Wilcoxon rank sum test which was used to compare control and treatment time to consume the first *Drosophila melanogaster* of pond E individuals.

p-value		Wilcoxon test statistic W	
Folpan WDG	Folpan SC	Folpan WDG	Folpan SC
0.507	0.485	15	13

Appendix A.4 – Scientific publication 4

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

Elena Adams, Christoph Leeb, Carsten A. Brühl

Abstract

Amphibian populations are declining worldwide at alarming rates. Among the large variety of contributing stressors, chemical pollutants like pesticides have been identified as a major factor for this decline. Besides direct effects on aquatic and terrestrial amphibian stages, sublethal effects like impairments in reproduction can affect a population. Therefore, we investigated the reproductive capacity of common toads (*Bufo bufo*) in the pesticide-intensive viticultural landscape of Palatinate in Southwest Germany along a pesticide gradient. In a semi-field study, we captured reproductively active common toad pairs of five breeding ponds with different pesticide contamination level and kept them in a net cage until spawning. Toads from more contaminated ponds showed an increased fecundity (more eggs) but decreased fertilization rates (fewer hatching tadpoles) as well as lower survival rates and reduced size in Gosner stage 25, suggesting that the higher exposed populations suffer from long-term reproductive impairments. In combination with acute toxicity effects, the detected sublethal effects, which are mostly not addressed in the ecological risk assessment of pesticides, pose a serious threat on amphibian populations in agricultural landscapes.

Keywords

Amphibians ● Semi-field study ● Fecundity ● Population decline ● Sublethal effects

Introduction

The latest IUCN reports suggest that 41% of all amphibian species are threatened (IUCN 2020). Besides habitat modification and destruction, intensive agriculture including the exposure to chemical pollutants like pesticides is one of the major factors for the global amphibian decline (Collins and Storfer 2003; Stuart et al. 2004). Several studies investigating the impact of intensive agriculture on amphibians determined adverse effects on egg and tadpole health (Babini et al. 2018), adult body condition, and morphology (Bionda et al. 2018; Hegde et al. 2019; Zhelev et al. 2017). One reason for these effects can be the exposure of amphibians to pesticides, with which they can come into contact during their whole life cycle. They can be exposed during the breeding phase and larval development in their aquatic habitats due to spray-drift (Crossland et al. 1982), run-off (Edwards et al. 1980) and drainages (Brown and van Beinum 2009). Post-metamorphic, terrestrial juvenile and adult amphibians can take up pesticides e.g., from contaminated soil (Storrs Méndez et al. 2009) during migration through

the agricultural landscape (Leeb et al. 2020b; Lenhardt et al. 2013). Despite this chronic, biphasic exposure, the effects of chemical pollutants on amphibian declines is not well understood (Grant et al. 2016). Most ecotoxicological laboratory studies on amphibians focus on acute effects of pesticides that lead to direct mortality in aquatic or, more rarely studied, terrestrial life stages (e.g., Brühl et al. 2013; Relyea 2004, 2005). Besides these acute effects, chronic and sublethal effects due to impaired reproduction may also result in amphibian population declines. Thus, there is not only a potential for rapid but also long-term amphibian declines, either due to impairment of adult breeding or deficient development of a progeny (Hayes et al. 2010b).

On the one hand, sublethal effects on reproduction can occur due to direct systemic toxicity. Effects on molecular biomarkers like acetylcholine esterase activities (Hegde et al. 2019) and hematological parameters (Zhelev et al. 2018) as well as genotoxic and mutagenic effects (Gonçalves et al. 2019) may have an impact on the reproductive capacity and thus on amphibian populations. Moreover, resources for the production of eggs may be limited and reproduction reduced due to resources required for pesticide detoxification processes as shown for the woodlouse *Porcellio scaber* (Jones and Hopkin 1998). Pesticides may also indirectly affect amphibian reproduction by interfering with their food supply (Sánchez-Bayo and Wyckhuys 2019) or affecting their behavior and thus disturbing their habitat use (Leeb et al. 2020a), predation (Adams et al. 2020), mating behavior (Schwendiman and Propper 2012) and population connectivity (Lenhardt et al. 2017).

On the other hand, pesticides can also directly act on the hormonal pathways of developmental processes as endocrine disrupting chemicals (EDCs), which alter the normal functioning of the endocrine system leading to impaired reproduction mechanisms such as infertility or intersex (Ujhegyi and Bókony 2020). EDCs have been found in amphibian breeding sites in agricultural landscapes. Bókony et al. (2018) detected 41 EDCs across amphibian ponds in the agricultural landscape of Hungary. Müller and Zithier (2015) performed a monitoring of ten pesticides in small water bodies used by amphibians in agricultural landscapes in North Germany and detected amongst others the potential EDCs metazachlor and propiconazole. However, in general little information on pesticide contamination is available on water bodies used by amphibians for spawning and larval development, as most studies investigate pollution of groundwaters, river systems and lakes (Lorenz et al. 2017), neglecting small, shallow water bodies that are especially important for amphibians (Wells 2007).

Studies on direct reproduction effects of pesticides on amphibians are considerably rare. One of the few well-studied pesticides with endocrine disruptive properties is the insecticide atrazine that shows severe effects on the reproduction of amphibians. Larvae of African clawed frogs (*Xenopus laevis*) showed a decreased gonadal volume and germ cells (Tavera-Mendoza et al. 2002a, b) as well as a trend to hermaphroditism (Hayes et al. 2002b) after exposure to atrazine. Further, atrazine induced feminization of male leopard frogs (*Lithobates pipiens*) in nature (Hayes et al. 2002a). Pesticide mixtures containing atrazine also indirectly inhibit reproductive functioning, e.g., by increasing stress hormone levels like corticosterone in adult *X. laevis* (Hayes et al. 2006). This may lead to further impacts including inhibition of sex hormones (Burmeister et al. 2001) and the alteration of reproductive development, breeding behavior and fertility (Moore 1983). Other current-use pesticides with endocrine disruptive properties are for example dicarboxamides like the viticultural fungicide vinclozolin (Kortekamp et al. 2011). This fungicide has been shown to contribute to shifted sex ratios, an inhibited maturation and reduced fecundity as well as fertility in fish (Lor et al. 2015). Although a few studies have explored endocrine disrupting effects of viticultural azole fungicides like tebuconazole and penconazole (e.g., Lv et al. 2017; Poulsen et al. 2015), they are not yet considered as EDCs by the Pesticide Properties DataBase (PPBD, Agriculture and Environment Research Unit of the University of Hertfordshire 2013) and the PAN International List of Highly Hazardous Pesticides (PAN List of HHPs; Pesticide Action Network International 2019). Further pesticides may have similar effects, however, the database on endocrine disruptive properties is too small to allow for concrete conclusions.

Especially field data on sublethal reproduction endpoints are scarce because mainly laboratory studies are used to investigate effects of pesticides on reproduction. Thereby, the most investigated endpoint in field studies analyzing effects on reproduction is the incidence of intersex, in which individual´s gonads contain both female and male tissue (Ujhegyi and Bókony 2020). However, also other endpoints like the number of laid eggs, fertilization rates or the development success of early larvae can be used to evaluate effects of pesticides on the reproductive capacity. Bókony et al. (2018) investigated the effects of EDCs on common toads (*Bufo bufo*) in agricultural and urbanized ponds in Hungary and observed reduced developmental rates and lower body mass of the offspring compared to natural ponds.

Investigations on pesticide effects on the reproduction of amphibians in viticultural landscapes do not exist so far, although viticulture is one of the most pesticide-intensive cultures in Central

Europe. On average 9.5 pesticide applications with a mixture of on average 1.6 formulations per application are performed during March and August in vineyards (Roßberg 2009). Because of the combined aquatic and terrestrial exposure of amphibians to viticultural pesticides, long-term adverse effects on reproduction are likely. To address this lack of knowledge, we investigated the reproductive capacity of common toads (*Bufo bufo*, LINNAEUS 1758) in the viticultural landscape of Palatinate in Southwest Germany along a pesticide gradient. We hypothesized that an increased chronic pesticide exposure affects fecundity, fertilization rate as well as offspring survival and size. Common toads were used since it is the most common amphibian species in Central Europe (Sillero et al. 2014) and it occupies a broad range of habitat types including agricultural landscapes like vineyards (Leeb et al. 2020b; Lenhardt et al. 2013). They are not yet considered endangered on an international as well as national level (Agasyan et al. 2009; Kühnel et al. 2009). However, population declines have been observed on a local level (e.g., Beebee and Griffiths 2005; Bonardi et al. 2011; Kyek et al. 2017; Petrovan and Schmidt 2016).

Materials and methods

Study sites and exposure assessment

In spring 2019, we studied common toad populations from five ponds (pond A–E, Table 1, Fig. 1) around Landau, one of the largest winegrowing areas in Southwest Germany. These ponds were expected to represent a gradient of pesticide contamination due to their varying agricultural surrounding. For validation of the pesticide gradient, five water samples were collected of each pond between April and May 2018 and analyzed for 47 different fungicides, six insecticides, three herbicides, and two acaricides (Table S1) by the Institute of Phytomedicine of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz in Neustadt/Weinstraße, Germany. The selection of analyzed pesticides was based on spraying recommendations for vine from local authorities (www.dlr.rlp.de).

Table 1 Locations of study ponds, contamination level (sum of toxic units, STU, see Equation 2), number of captured toad pairs and number of toad pairs that spawned

Pond	Coordinates (WGS84)	STU	Number of toad pairs	
			Captured	Spawned
A	49.25475, 7.96182	-4.48	12	11
B	49.23830, 7.99002	-3.48	13	11
C	49.20329, 8.20917	-3.09	15	13
D	49.21830, 8.04944	-2.25	14	14
E	49.18898, 8.03709	-1.75	8	5

Pond letters indicate increasing STU. Since no pesticides were detected in pond A, its STU was calculated based on the use of 1/10 of the minimum TU observed in the sites with detected concentrations (for rationale s. Schäfer et al. 2011)

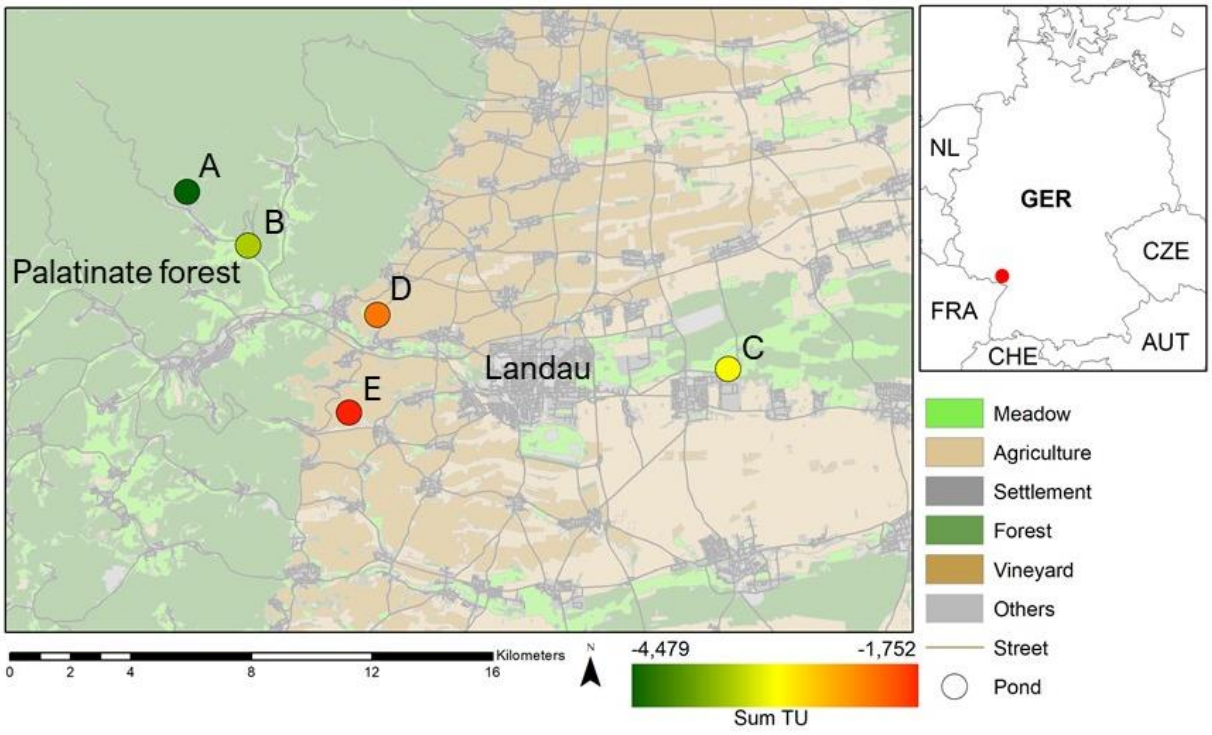


Fig. 2 Map of study ponds in Palatinate in Southwest Germany. Increasing letters and colors of study sites represent the pesticide contamination from no contamination (dark-green, pond A) to high contamination (red, Pond E). Source: Basemap: DLM50 - ©GeoBasis-DE / LVermGeoRP2020, dl-de/by-2-0, www.lvermgeo.rlp.de [modified data].

The pond pesticide toxicity was assessed using Toxic Units for each detected pesticide (Eq. 1, with C_i = detected concentration of pesticide i and $LC50_i$ = median lethal concentration causing 50% mortality of test organisms).

$$TU = \frac{C_i}{LC50_i} \quad (1)$$

As LC50 values for amphibians are often lacking, data of acute fish toxicity studies compiled from the PPDB (Agriculture and Environment Research Unit of the University of Hertfordshire 2013) were used as proxy for amphibians (Weltje et al. 2013). The sum of TU (STU, Eq. 2, with n = number of detected pesticides) was calculated to aggregate the toxicity of the detected pesticides (Table 1, Schäfer et al. 2011) by using the maximum detected sum of TU of each study pond. To allow the comparison to sites without any detected pesticides, uncontaminated ponds were assigned to a TU of 1/10 of the minimum TU observed in the contaminated sites (Fernández et al. 2015), leading to a STU of −4.48 for pond A.

$$STU = log(max \sum_{i=1}^{n} TU) \quad (2)$$

The detected pesticides were checked for endocrine disruptive properties using toxicity data from the PPDB (Agriculture and Environment Research Unit of the University of Hertfordshire 2013) and the PAN List of HHPs (Pesticide Action Network International 2019). Moreover, acute and chronic regulatory acceptable concentrations (RACs) were calculated based on fish toxicity values from the PPDB (LC50 and NOEC = No observed effect concentration, Eqs. 3, 4, Table S2). As uncertainty factors, 100 was used for the acute and 10 for the chronic RAC as recommended for aquatic organisms by EFSA (2013). The RACs were compared to the detected concentrations to estimate the acute and chronic aquatic toxicity of the ponds.

$$RAC_{acute} = \frac{LC50}{100} \quad (3)$$

$$RAC_{chronic} = \frac{NOEC}{10} \quad (4)$$

Moreover, the landscape composition around the study ponds was analyzed. Based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50), the percentages of vineyards, other

agriculture, meadows, settlements, and forests were calculated. A radius of three kilometer was chosen to analyze the landscape composition because this distance reflects the annual migrations between hibernation as well as summer habitats and breeding ponds for *B. bufo* (Günther 2009). To estimate the terrestrial exposure, data of viticultural and other agricultural area was used.

Reproductive capacity analysis

We aimed to capture ten or more reproductively active adult common toad pairs during their spawning season between 9 and 28 March 2019 from each pond. After capturing, each pair was housed in a net cage (80 × 65 × 60 cm) in the respective breeding pond containing a wire hanger as spawning substrate. Due to the short spawning season of *B. bufo* and the fact that not all pairs spawned, it was not possible to investigate ten spawning pairs of each pond (Table 1). Finally, we captured 62 toad pairs from which eight pairs did not spawn, 45 pairs spawned within 7 days and nine pairs within 15 days after catchment. One day after spawning, the body mass of each toad was measured (±0.1 g) and the individuals were released in their ponds. It can be assumed that females laid all eggs at once because the spawning process is usually finished after 6 to 12 h (Günther 2009) and the pairs terminated the amplexus after oviposition.

As measures of each population's reproductive capacity, we analyzed the fecundity, fertilization rate, offspring survival until the free-swimming Gosner Stage 25 (GS; Gosner 1960) and offspring size (tadpole length) at GS25. To determine the fecundity, the number of laid eggs per female was counted. Because fecundity is known to increase with female size (Banks and Beebee 1986; Reading 1986), we calculated the ratio of the amount of laid eggs and the body mass of the females after spawning (eggs/g body mass). To estimate the fertilization rate and offspring survival, approximately 90 eggs of each clutch were removed from three randomly chosen parts of the egg string and kept individually in clear plastic aquariums (22.5 × 16.5 × 7 cm, Braplast, Bergheim, Germany) filled with 1 L FETAX medium (Dawson and Bantle 1987). To prevent any injuries of eggs, the handling of the spawning strings was kept to a minimum. Thus, the number of eggs was not identical for each sample. Because mold grew on the first three egg strings collected from pond C, three samples of pond C could not be used to analyze the fertilization rate and offspring survival. To prevent mold from growing on further eggs, eggs of one egg string were separated but still incubated together in one aquarium. The eggs were reared in a climate chamber at 21 ± 1 °C and a 16:8 h day:night light cycle until they reached GS25. The individuals were photographed daily. Three days after

spawning, non-fertilized eggs that exhibited mold growing on them or did not show embryonic development were removed. Developing eggs were counted using Image J (Schneider et al. 2012) to calculate the fertilization rate. Fertilized eggs from one egg string hatched within a time difference of maximum 24 h. As soon as all tadpoles reached GS25 (9–10 days), the proportion of embryos that survived to this stage was counted to estimate the offspring survival. Moreover, the lengths of twelve randomly selected tadpoles of each sample were determined to estimate the offspring's sizes. After recording the needed data, the tadpoles were released in their origin pond.

Statistical analyses

Statistical analyses were performed using R (version 3.5.2; R Core Team 2013). To determine the correlation of the aquatic and terrestrial exposure, a Pearson´s correlation was performed. Kendall-Theil Sen Siegel non-parametric regressions (Sen 1968; Siegel 1982; Theil 1950) were performed to check whether the investigated endpoints depend on the pesticide contamination of ponds (STU). Moreover, Spearman's rank correlations between the investigated endpoints and the STU of ponds were computed (Spearman's rank correlation coefficient ρ, Hollander et al. 1973).

To check the assumption that fecundity is increased by female size, a Spearman's rank correlation was performed for the female body mass and the number of laid eggs. Moreover, Spearman's rank correlations were performed to investigate the relationship between the pesticide contamination (STU) and the female body mass, the number of laid eggs and the tadpole length in GS25, parental body masses and the fertilization rate as well as the number of laid eggs per female and the fertilization rate. To investigate a measure of population fitness, the product of the four investigated reproductive endpoints was calculated and a one-way analysis of variance (ANOVA) was performed to identify differences between the investigated ponds. Tukey's method was used to identify and remove outliers ranged above and below the $1.5 \times \text{IQR}$ (Kannan Senthamarai et al. 2015). For all statistical tests, the criterion for significance was set to $\alpha = 0.05$.

Results

Exposure assessment

The pesticide residue analysis revealed 22 different pesticides in total and 0–19 different pesticides per pond with a STU between −4.48 and −1.75 (Tables 1, S2) meaning no aquatic toxicity at a STU of −4.48 and high toxicity at a STU of −1.75. Toxicity data extracted from the PPDB and the PAN List of HHPs for the detected pesticides did not show any endocrine disruptive properties or the data base was insufficient to make a statement about endocrine disruptive properties. However, azole fungicides which were shown to be potential EDCs (Kortekamp et al. 2011; Lv et al. 2017; Poulsen et al. 2015) were detected in the ponds. Penconazole was detected in ponds B, D and E (0.02–0.18 µg/L), tebuconazole in ponds C, D and E (0.05–0.08 µg/L) and difenconazole in pond C (0.02 µg/L).

The comparison of detected concentrations to RACs revealed a conspicuous toxicity of the chronic exposure to the fungicides folpet and famoxadone and the acute exposure to famoxadone in pond E (Table S2). The chronic RAC of folpet was 5.6 times lower than the detected concentration in sampling 2 (4.53 µg/L), the chronic RAC of famoxadone was 1.1 times lower and the acute RAC of famoxadone was 1.4 times lower than the detected concentration in sampling 5 (0.15 µg/L), resulting in an increased hazard of adverse effects.

The landscape composition analysis showed an increasing agricultural land-use from pond A to pond E in a three-kilometer radius around the study ponds ranging from 0 to 60% (Table 2). The Pearson correlation revealed a statistically significant correlation between the STU and the agricultural land-use ($p = 0.02$, Pearson's $r = 0.94$, df = 3).

Table 2. Landscape composition in a radius of 3000 m around the study ponds based on a vector landscape model of Rhineland-Palatinate (ATKIS DLM50)

Pond	Viticulture [%]	Other agriculture [%]	Meadow [%]	Settlement [%]	Forest [%]	Other [%]
A	0.0	0.0	5.1	1.3	92.9	0.6
B	0.1	1.1	19.2	5.6	72.1	1.9
C	0.3	31.4	19.6	15.5	28.5	4.8
D	47.5	1.1	7.9	11.6	29.8	2.2
E	57.0	3.1	6.1	10.1	22.5	1.3

Reproductive capacity

Neither the female body mass (52.0 ± 14.1 g), the male body mass (33.46 ± 6.7 g), nor the number of laid eggs per female (3243 ± 1538) affected the fertilization rate ($\rho = -0.24$, $p = 0.10$, $\rho = -0.09$, $p = 0.56$ and $\rho = -24$, $p = 0.10$). The female body mass was positively correlated with the number of laid eggs ($\rho = 0.62$, $p < 0.001$) and the STU ($\rho = 0.38$, $p < 0.01$). Moreover, the offspring size (tadpole length in GS25) was negatively correlated with the number of laid eggs per female ($\rho = -0.32$, $p = 0.03$).

Kendall-Theil Sen Siegel regressions revealed a significant influence of the STU on all investigated endpoints ($p < 0.001$, Table S3). The mean fecundity differed from 49 to 74 eggs/g body mass and showed a positive correlation with increasing STU ($\rho = 0.54$, $p < 0.001$, Fig. 2A, Table S4). The fertilization rate, offspring survival and tadpole lengths showed mean decreases of 4.5%, 32.6% and 10.7% with increasing STU (Fig. 2A–D, Table S4). Negative correlations between the STU and the fertilization rate ($\rho = -0.32$, $p = 0.03$, Fig. 2B), the offspring survival ($\rho = -0.57$, $p < 0.001$, Fig. 2C) as well as the offspring size ($\rho = -0.49$, $p < 0.001$, Fig. 2D) were observed. The performed ANOVA did not reveal any differences for population fitness between the study ponds ($p > 0.05$).

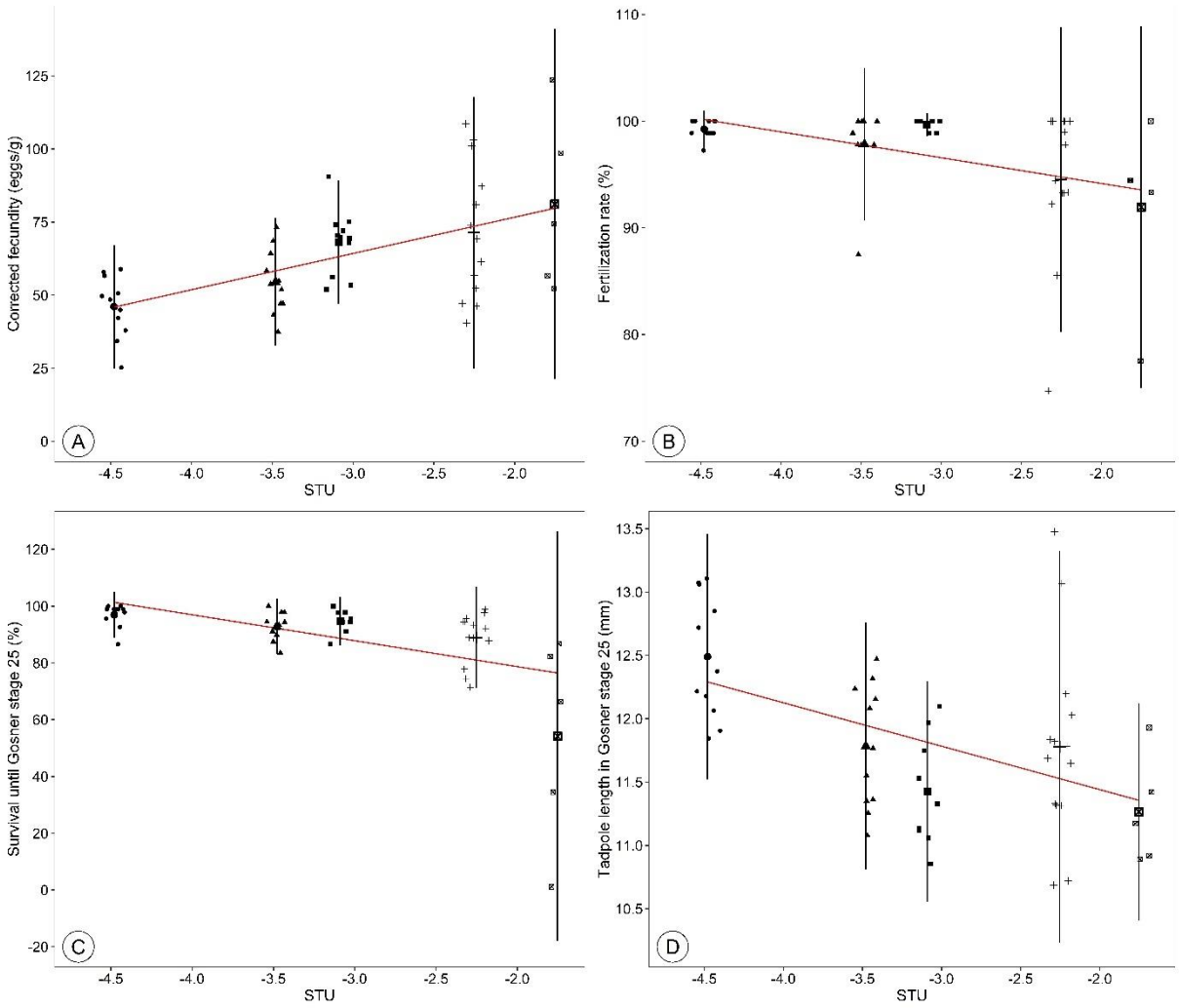


Fig. 2 Dependence of fecundity (**A**), fertilization rate (**B**), offspring survival until Gosner stage 25 (**C**) and offspring size in Gosner stage 25 (**D**) on the pesticide contamination of breeding ponds (maximum sum of toxic units, STU). Fecundity was corrected for the body mass of the females after spawning (eggs/g body mass). For each pond, the means and standard deviations are presented (Table S4)

Discussion

Exposure assessment

Since pesticide contamination of ponds are often reported to correlate with the surrounding agricultural land-use (Baker 2006), it was assumed that the detected pesticide gradient also represents the exposure during the pre- and post-breeding migration of the terrestrial amphibian stages. The determined correlation of aquatic exposure and land-use confirms this hypothesis.

No general statement can be drawn about the endocrine disruptive potential of the detected pesticides because further research is needed on their potential to act as EDCs. The well-studied endocrine disrupting herbicide atrazine was not detected in any of the study ponds probably because it is prohibited in Germany since 1991. However, since potentially endocrine disruptive pesticides like the azole fungicides penconazole, tebuconazole and difenconazole were detected, similar endocrine effects are likely. Furthermore, the ponds were only analyzed for active ingredients of pesticides. A statement about the toxicity of product additives, which can have a high acute toxicity, endocrine disruptive or reproductive toxic properties themselves or as metabolite (Mesnage and Antoniou 2017; Mullin et al. 2016), cannot be made.

The comparison of detected concentrations to chronic RACs of folpet and famoxadone in pond E reveals a high toxicity for aquatic vertebrates. Next to possible adverse effects because of single pesticides, mixture effects in ponds with up to 19 detected pesticides may contribute to higher toxicities (Relyea 2009). Moreover, it cannot be excluded that even higher concentrations and further pesticides were present in the ponds due to the limited number of water samplings ($n = 5$) and analyzed pesticides ($n = 58$ target molecules). Since only one rain event sampling was performed in the present study, peak pesticide concentrations may be underestimated (Neumann et al. 2003). Especially folpet and famoxadone may be present at higher concentrations than detected because they have very short dissipation times in water (DT50 folpet = 0.02 d, DT50 famoxadone = 0.1 d, Agriculture and Environment Research Unit of the University of Hertfordshire 2013).

Reproductive capacity

Toads of the highest contaminated pond E showed on average a 1.5 times higher fecundity than toads of the uncontaminated pond A. In comparison to the present study, Bókony et al. (2018) did not observe any effect on the fecundity of common toads in agricultural ponds compared to natural ponds. Because the female body mass correlated with the number of eggs and both of them correlated with STU, the increased fecundity may be based on the higher female body masses in the contaminated ponds. Guillot et al. (2016) also observed larger and heavier common toads in French agricultural habitats compared to uncontaminated forest habitats. The increased body sizes might either suggest a potential adjustment during aging or some habitat specificities in the agricultural landscape may enhance body size. For example, smaller population densities in agricultural landscapes might decrease intra- and/or interspecific competition leading to larger individuals (Bishop et al. 1999; Guillot et al. 2016; Janin et

al. 2011). However, there are multiple reasons that may affect adult body size without an agricultural context such as climate, habitat geography, size at metamorphosis, and availability of food resources.

The fertilization rate was negatively affected with increasing pesticide contamination of the ponds, suggesting that the higher exposed populations suffer from long-term reproductive impairments. There are several reasons that may have led to the observed decreased fertilization rate. Due to the increased number of eggs per female, the male fertilization success may be reduced. But also behavioral impairments during mating could lead to decreased fertilization rates. Hayes et al. (2010a) observed a reduced success of amplexus in male *X. laevis* exposed to atrazine and thus a lower proportion of fertilized eggs for atrazine exposed males. Also endocrine disruptive properties of pesticides may have led to this decrease for example due to impaired spermatogenesis which already has been reported after the exposure of frogs to the herbicide atrazine. Hayes et al. (2010a) observed a decreased frequency of testicular tubules with mature spermatozoa in *X. laevis*. In *X. laevis* tadpoles a reduction in testicular volume during sexual differentiation of the testis was observed (Tavera-Mendoza et al. 2002b). Another reason may be an effect on female sexual development. In-vitro assays with eleven pesticides of Orton et al. (2009) revealed altered ovarian steroidogenesis and reduced progesterone production. Pickford and Morris (2003) investigated the effects of the insecticide methoxychlor on female *X. laevis* and detected an inhibition of oviposition and maturation of oocytes. Moreover, the exposure to atrazine caused a reduction in the number of germ cells in the ovary and an increase of damaged oocytes (Tavera-Mendoza et al. 2002b). The larval exposure of *X. laevis* to atrazine induced a reduction of testosterone levels in males (Hayes et al. 2010a) leading to a decrease of male reproductive success (Moore and Hopkins 2009).

Decreasing survival rates and tadpole sizes were observed with increasing pesticide contamination. Bókony et al. (2018) also observed reduced body masses of common toad larvae and juveniles in agricultural landscapes in comparison to natural landscapes. Clearly, decreased survival of the tadpoles directly leads to population declines. The reduced tadpole lengths could lead to further impairments since body size is a critical determinant of individual fitness (Wells 2007). Smaller tadpoles sizes lead to reduced sizes at metamorphosis and thus to a decreased survivorship of the first hibernation (Üveges et al. 2016) and until maturity as well as delayed achievement of reproductive size (Smith 1987). Reduced body size is also a

disadvantage as adult for reproduction because it affects female fecundity and male mating success (Banks and Beebee 1986; Davies and Halliday 1979; Reading et al. 1991).

On the one hand, reduced offspring size may be a long-term consequence of chronic pesticide pollution over several generations. Transgenerational effects were observed in rats after the exposure to EDCs as Anway et al. (2005) detected a decreased spermatogenic capacity in cell number and viability as well as an increase of male infertility in four tested generations. Thus, early-life exposure of parents can lead to impaired offspring viability. To verify the proposed reasons of reproduction impairments regarding endocrine disruptive effects, tissue analyses of e. g. thyroids and gonads would be needed. However, the present study was designed and completed without any lethal interferences and tissue withdrawals of the amphibian populations.

On the other hand, the reduced offspring size originating from highly contaminated ponds may be a cost of an evolutionary adaptive resistance (Whitehead et al. 2012) or of detoxification processes of contaminants (Rix et al. 2016). Similar effects have been observed for urban fish populations which evolved tolerance to toxic pollutants (Meyer and Di Giulio 2003; Whitehead et al. 2012). However, their offspring showed reduced growth rates and were more susceptible to other stressors compared with the offspring from a non-contaminated site (Meyer and Di Giulio 2003). Similar trade-offs may be responsible for the smaller tadpoles of the more contaminated ponds. Adult toads of these ponds may invest more resources into the production of egg jelly coat material to provide a better protection against pesticides. These resources may have in turn not be invested into larger ova (Podolsky 2004) which may have led to smaller tadpoles such as determined by Kaplan (1980). The higher egg production in contaminated ponds may be discussed as an adaptation to increase fitness by counterbalancing negative pesticide effects on embryo and tadpole development by an increased egg number.

Although amphibians are especially affected by pesticides due to their biphasic lifecycle, they are not yet considered in the environmental risk assessment of pesticides in the EU (Ockleford et al. 2018). Our data support the suggestion of inhibitory effects of current-use pesticides on the reproductive capacity of amphibians, potentially contributing to population declines. Thus, not only acute effects should be investigated in ecotoxicological amphibian studies but also sublethal effects on reproduction on a population level. Since data involving field scenarios

analyzing the effects of multiple pesticides on amphibian reproduction are considerably rare, our results are of significant importance for amphibian conservation in agricultural landscapes.

Data availability

Data are available by contacting EA (adams@uni-landau.de).

Code availability

The used R code is available by contacting EA (adams@uni-landau.de).

Acknowledgements

We thank Andreas Kortekamp and Michael Twertek of the Dienstleistungszentrum Ländlicher Raum Rheinpfalz for pesticide residue analysis. Sincere thanks to Stephan Adams for the assistance during the study. Moreover, the authors are grateful for the valuable comments of Verena Gerstle on earlier drafts of this paper.

Funding

The work was financed by the Ph.D. scholarship program of the German Federal Environmental Foundation. CL was financed by the Deutsche Forschungsgemeinschaft (DFG-TH 498 1807-2). Open Access funding enabled and organized by Projekt DEAL.

Author contributions

All authors conceived and designed the study. EA performed the study, analyzed the data and drafted the manuscript. CL generated the map. CL and CAB contributed to and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the Struktur- und Genehmigungsdirektion Süd (Neustadt an der Weinstraße, Germany, license number 42/553-254/457-19).

Supplementary information

The online version of this article (https://doi.org/10.1007/s10646-020-02335-9) contains supplementary material, which is available to authorized users.

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Supporting information of scientific publication 4

Supplementary material Table S1

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

Elena Adams[1*], Christoph Leeb[1], Carsten A. Brühl[1]

[1]iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

*Corresponding author: adams@uni-landau.de

Table S1. Investigated target pesticides of the aquatic residual analysis. In total 47 different fungicides, six insecticides, three herbicides and two acaricides were investigated.

Fungicides	Insecticides	Herbicides	Acaricides
Amisulbrom	Chlorpyrifos-methyl	Atrazine	Spirodiclofen
Azoxystrobin	Dimethoate	Carfentrazone-ethyl	Tebufenpyrad
Benalaxyl-M	Indoxacarb	Simazine	
Benthivalicarb	Methidathion		
Boscalid	Parathion-ethyl		
Captan	Parathion-methyl		
Cyazofamid			
Cyflufenamid			
Cyprodinil			
Dichlofluanid			
Difenoconazole			
Dimethomorph			
Epoxiconazole			
Famoxadone			
Fenarimol			
Fenhexamid			
Fenpropimorph			
Fenpyrazamine			
Folpet			
Fludioxonil			
Fluopicolide			

Fluopyram			
Fluquinconazole			
Iprodion			
Iprovalicarb			
Kresoxim-methyl			
Metalaxyl M			
Metrafenone			
Myclobutanil			
Penconazole			
Prochloraz			
Procymidon			
Propinconazole			
Proquinazid			
Pyraclostrobin			
Pyrifenox			
Pyrimethanil			
Quinoxyfen			
Spiroxamin			
Tebuconazole			
Tetraconazole			
Tolylfluanid			
Triadimefon			
Triadimenol			
Trifloxystrobin			
Vinclozolin			
Zoxamide			

Supplementary material Table S2

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

Elena Adams[1*], Christoph Leeb[1], Carsten A. Brühl[1]

[1]iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

*Corresponding author: adams@uni-landau.de

Table S2. Detected pesticide concentrations in the aquatic residual analysis of each study pond and respective risk assessment parameters. In total five samplings were performed (06.04.2018, 19.04.2018, 03.05.2018, 10.05.2018 - rain event, 24.05.2018). Pond letters indicate increasing pesticide contamination (based on the sum of toxic units).

DC = Detected concentration; NOEC = No observed effect concentration (chronic toxicity) for fish and LC50 = Median lethal concentration causing 50% mortality of fish (acute toxicity) were extracted from the Pesticide Properties Database (Agriculture and Environment Unit of the University of Hertfordshire 2013); NOEC-RAC = Regulatory acceptable concentration based on the NOEC values, which are divided by the assessment factor for chronic studies (10); LC50-RAC = Regulatory acceptable concentration based on the LC50 values, which are divided by the assessment factor for acute studies (100). Detected concentrations were divided by calculated RAC values. A result > 1 (highlighted in bold) reveals a possible hazard for aquatic organisms.

Sampling	**Pesticide**	**DC (µg/L)**	**NOEC (µg/L)**	**NOEC-RAC (µg/L)**	**DC/NOEC-RAC**	**LC50 (µg/L)**	**LC50-RAC (µg/L)**	**DC/LC50-RAC**
Pond A								
1-5	-	-	-	-	-	-	-	-
Pond B								

1	Boscalid	0.09	125	12.5	0.0072	2700	27	0.0033
	Fludioxonil	0.04	40	4	0.0100	230	2.3	0.0174
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
2	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Zoxamide	0.03	4	0.4	0.0750	160	1.6	0.0188
3	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
4	Fludioxonil	0.06	40	4	0.0150	230	0.23	0.26
	Myclobutanil	0.14	200	20	0.0070	2000	2	0.07
	Dimethoate	0.02	400	40	0.0005	30200	30.2	0.0007
5	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Penconazole	0.02	360	36	0.0006	1130	11.3	0.0018
Pond C								
1	Azoxystrobin	0.04	147	14.7	0.0027	470	4.7	0.0085
	Difenconazole	0.02	23	2.3	0.0087	1100	11	0.0018
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Iprovalicarb	0.28	9890	989	0.0003	22700	227	0.0012
	Metalaxyl M	0.08	9100	910	0.0001	27000	270	0.0003
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Dimethoate	0.05	400	40	0.0013	30200	302	0.0002
2	Azoxystrobin	0.03	147	14.7	0.0020	470	4.7	0.0064

	Fluopyram	0.02	135	13.5	0.0015	42900	429	0.0000
	Zoxamide	0.12	4	0.4	0.3000	160	1.6	0.0750
3	Folpet	0.33	8.1	0.81	0.4074	680	6.8	0.0485
	Iprovalicarb	0.09	9890	989	0.0001	22700	227	0.0004
4	Azoxystrobin	0.02	147	14.7	0.0014	470	4.7	0.0043
	Fludioxonil	0.05	40	4	0.0125	230	2.3	0.0217
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.04	200	20	0.0020	2000	20	0.0020
	Pyrimethanil	0.02	1600	160	0.0001	10560	105.6	0.0002
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
5	Cyflufenamid	0.02	24	2.4	0.0083	1040	10.4	0.0019
	Folpet	0.22	8.1	0.81	0.2716	680	6.8	0.0324
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Metrafenone	0.10	228	22.8	0.0044	820	8.2	0.0122
	Myclobutanil	0.10	200	20	0.0050	2000	20	0.0050
	Tebuconazole	0.07	10	1	0.0700	4400	44	0.0016
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.04	400	40	0.0010	30200	302	0.0001
Pond D								
1	Dimethomorph	0.06	56	5.6	0.0107	6100	61	0.0010

	Famoxadone	0.06	1.4	0.14	0.4286	11	0.11	0.5455
	Iprovalicarb	0.44	9890	989	0.0004	22700	227	0.0019
	Metalaxyl M	0.08	9100	910	0.0001	27000	270	0.0003
	Myclobutanil	0.22	200	20	0.0110	2000	20	0.0110
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
2	Boscalid	0.04	125	12.5	0.0032	2700	27	0.0015
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Zoxamide	0.04	4	0.4	0.1000	160	1.6	0.0250
3	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
4	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Cyflufenamid	0.02	24	2.4	0.0083	1040	10.4	0.0019
	Fludioxonil	0.19	40	4	0.0475	230	2.3	0.0826
	Fluopyram	0.17	135	13.5	0.0126	42900	429	0.0004
	Iprovalicarb	0.12	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.06	200	20	0.0030	2000	20	0.0030
	Penconazole	0.18	360	36	0.0050	1130	11.3	0.0159
	Pyrimethanil	0.04	1600	160	0.0003	10560	105.6	0.0004
	Tebuconazole	0.08	10	1	0.0800	4400	44	0.0018
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.08	400	40	0.0020	30200	302	0.0003
5	Famoxadone	0.05	1.4	0.14	0.3571	11	0.11	0.4545

	Fludioxonil	0.06	40	4	0.0150	230	2.3	0.0261
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
Pond E								
1	Azoxystrobin	0.07	147	14.7	0.0048	470	4.7	0.0149
	Boscalid	0.27	125	12.5	0.0216	2700	27	0.0100
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Fluopyram	0.21	135	13.5	0.0156	42900	429	0.0005
	Iprovalicarb	0.12	9890	989	0.0001	22700	227	0.0005
	Metrafenone	0.21	228	22.8	0.0092	820	8.2	0.0256
	Tetraconazol	0.02	300	30	0.0007	4400	44	0.0005
2	Boscalid	0.02	125	12.5	0.0016	2700	27	0.0007
	Dimethomorph	0.1	56	5.6	0.0179	6100	61	0.0016
	Folpet	4.53	8.1	0.81	**5.5926**	680	6.8	0.6662
	Fluopicolide	0.04	155	15.5	0.0026	360	3.6	0.0111
	Fluopyram	0.13	135	13.5	0.0096	42900	429	0.0003
	Iprovalicarb	3.05	9890	989	0.0031	22700	227	0.0134
	Kresoxim-methyl	0.22	13	1.3	0.1692	190	1.9	0.1158
	Metrafenone	0.05	228	22.8	0.0022	820	8.2	0.0061

	Myclobutanil	0.73	200	20	0.0365	2000	20	0.0365
	Penconazole	0.03	360	36	0.0008	1130	11.3	0.0027
	Pyrimethanil	0.03	1600	160	0.0002	10560	105.6	0.0003
	Zoxamide	0.14	4	0.4	0.3500	160	1.6	0.0875
	Dimethoate	0.06	400	40	0.0015	30200	302	0.0002
3	Folpet	0.58	8.1	0.81	0.7160	680	6.8	0.0853
	Fludioxonil	0.07	40	4	0.0175	230	2.3	0.0304
	Fluopyram	0.20	135	13.5	0.0148	42900	429	0.0005
	Dimethoate	0.02	400	40	0.0005	30200	302	0.0001
4	Boscalid	0.15	125	12.5	0.0120	2700	27	0.0056
	Folpet	0.20	8.1	0.81	0.2469	680	6.8	0.0294
	Fludioxonil	0.22	40	4	0.0550	230	2.3	0.0957
	Fluopyram	0.18	135	13.5	0.0133	42900	429	0.0004
	Iprovalicarb	0.11	9890	989	0.0001	22700	227	0.0005
	Myclobutanil	0.03	200	20	0.0015	2000	20	0.0015
	Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049
	Dimethoate	0.03	400	40	0.0008	30200	302	0.0001
5	Boscalid	0.26	125	12.5	0.0208	2700	27	0.0096
	Famoxadone	0.15	1.4	0.14	**1.0714**	11	0.11	**1.3636**
	Folpet	0.73	8.1	0.81	0.9012	680	6.8	0.1074
	Fludioxonil	0.25	40	4	0.0625	230	2.3	0.1087

Fluopicolide	0.53	155	15.5	0.0342	360	3.6	0.1472
Fluopyram	0.23	135	13.5	0.0170	42900	429	0.0005
Iprovalicarb	0.36	9890	989	0.0004	22700	227	0.0016
Metalaxyl M	0.24	9100	910	0.0003	27000	270	0.0009
Metrafenone	0.3	228	22.8	0.0132	820	8.2	0.0366
Myclobutanil	0.24	200	20	0.0120	2000	20	0.0120
Tebuconazole	0.05	10	1	0.0500	4400	44	0.0011
Zoxamide	0.02	4	0.4	0.0500	160	1.6	0.0125
Chlorpyrifos-methyl	0.02	5	0.5	0.0400	410	4.1	0.0049

Supplementary material Table S3

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

Elena Adams[1*], Christoph Leeb[1], Carsten A. Brühl[1]

[1]iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

*Corresponding author: adams@uni-landau.de

Table S3. Results of the Kendall-Theil Sen Siegel regression model to identify whether the pesticide contamination of ponds (sum of toxic units, STU) affects the investigated reproduction endpoints.

	Coefficient	Estimate	df	*p*
Fecundity	STU	13.52	50	< 0.001
Fertilization rate	STU	-0.24	46	< 0.001
Offspring survival	STU	-3.93	46	< 0.001
Offspring fitness	STU	-0.04	48	< 0.001

Supplementary material Table S4

Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape

Elena Adams[1*], Christoph Leeb[1], Carsten A. Brühl[1]

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*Corresponding author: adams@uni-landau.de

Table S4. Determined median, mean and standard deviation for the investigated reproductive endpoints and ponds.

Endpoint	Pond	Median	Mean	Standard deviation
Fecundity (eggs/g)	A	46	49	11
	B	55	54	11
	C	68	70	11
	D	71	69	23
	E	81	74	30
Fertilization rate (%)	A	99.2	98.9	0.9
	B	97.8	98.96	3.6
	C	99.7	100.0	0.5
	D	94.5	96.1	7.1
	E	92.0	94.4	8.5
Offspring survival (%)	A	97.0	98.9	4.1
	B	92.9	93.3	4.8
	C	94.7	95.0	4.2
	D	88.9	92.1	8.9
	E	54.2	66.3	36.0
Tadpole length (mm)	A	12.5	12.4	0.5
	B	11.8	11.8	0.5
	C	11.4	11.3	0.4
	D	11.8	11.7	0.8
	E	11.3	11.2	0.4

Appendix A.5 – Declaration

I hereby declare that I independently conducted the work presented in this thesis entitled "**Pesticide effects on German amphibians and consequences for their risk assessment in the European Union**". All used resources and references are specified in this work. This thesis has not been submitted in any form elsewhere for a scientific examination, as a thesis or for evaluation in a similar context to any department of this University or any other scientific institution. I did not use the assistance of a doctoral consultant (or a similar person) in return for payment. All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective scientific publication. Contributions of me and the co-authors are given below.

Appendix A.1:

Adams, E., Leeb, C., Roodt, A.P., Brühl, C.A. (2021). Interspecific sensitivity of European amphibians towards two pesticides and comparison to standard test species. Environmental Sciences Europe 33:49. DOI: 10.1186/s12302-021-00491-1

Contributions:

Elena Adams (85%): Conceptualization, Methodology, Investigation, Formal analysis, Writing (Original draft preparation)
Christoph Leeb (5%): Conceptualization, Writing (Reviewing and editing)
Alexis Pieter Roodt (5%): Pesticide residue analysis, Writing (Reviewing and editing)
Carsten A. Brühl (5%): Conceptualization, Writing (Reviewing and editing)

Appendix A.2:

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. DOI: 10.1016/j.scitotenv.2021.147865

Contributions:

Elena Adams (85%): Conceptualization, Methodology, Investigation, Formal analysis, Writing (Original draft preparation)
Verena Gerstle (5%): Investigation, Writing (Reviewing and editing)
Tobias Schmitt (5%): Investigation, Writing (Reviewing and editing)
Carsten A. Brühl (5%): Conceptualization, Writing (Reviewing and editing)

Appendix A.3:

Adams, E., Gerstle, V., Brühl, C.A. (2021). Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*). Environmental Toxicolology and Chemistry 50(5), 1289-1297. DOI: 10.1002/etc.4972

Contributions:

Elena Adams (65%): Conceptualization, Methodology, Investigation, Formal analysis, Writing (Original draft preparation)

Verena Gerstle (30%): Conceptualization, Methodology, Investigation, Writing (Reviewing and editing)

Carsten A. Brühl (5%): Conceptualization, Writing (Reviewing and editing)

Appendix A.4: Adams, E., Leeb, C., Brühl, C.A. (2021). Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape. Ecotoxicology 30, 213-223. DOI: 0.1007/s10646-020-02335-9

Contributions:

Elena Adams (90%): Conceptualization, Methodology, Investigation, Formal analysis, Writing (Original draft preparation)

Christoph Leeb (5%): Conceptualization, Writing (Reviewing and editing)

Carsten A. Brühl (5%): Conceptualization, Writing (Reviewing and editing)

I am aware that a violation of the above-mentioned points can lead to a withdrawal of the doctoral degree and legal consequences.

Place, Date

Elena Adams

Appendix A.6 – Curriculum Vitae

Personal information

Name	Elena Adams
E-Mail	El.Adams@web.de
Date of birth	13.03.1992
Nationality	Germany

Scientific education

Since 01/2018	**Dissertation in "Environmental Sciences" (Ph.D.)** University of Koblenz-Landau, Landau Thesis: "*Pesticide effects on German amphibians and consequences for their risk assessment in the European Union*"
2014 - 2016	**Master program "Ecotoxicology" (M.Sc.)** University of Koblenz-Landau, Landau Thesis: "*Effects of the fungicide folpet on early developmental stages of Rana temporaria"* Graduation grade: 1.5
2011 - 2014	**Bachelor program "Forensic Sciences" (B.Sc.)** University of Applied Sciences Bonn-Rhein-Sieg, Rheinbach Thesis: "*Analytische Freigabeprüfungen eines Arzneitees"* Graduation grade: 2.0
2002 - 2011	**Allgemeine Hochschulreife** Megina-Gymnasium Mayen Graduation grade: 1.9

Work experience

Since 10/2020	**Ecotoxicology expert for terrestrial vertebrates** Bayer AG, Research & Development, Division Crop Science, Monheim
05/2017 – 12/2017	**Research assistant** BLE project „MaNaKa: Koordination und Erstellung eines Maßnahmenkatalogs für einen erfolgreichen Besatz des bedrohten heimischen Edelkrebses“ University of Koblenz-Landau, Landau
08/2016 – 10/2016 and 02/2017 – 04/2017	**Student assistant** DFG project “Amphibians in agricultural landscapes: chemical landscape fragmentation implemented in a habitat modelling approach” University of Koblenz-Landau, Landau
06/2015 – 08/2015	**Intern** Aquatic ecotoxicology laboratory of Bayer AG, Research & Development, Division Crop Science, Monheim
03/2014 – 06/2014	**Intern** Quality control of Sidroga Gesellschaft für Gesundheitsprodukte mbH, Bad Ems

Publications and presentations

Scientific publications included in this thesis

Adams, E., Leeb, C., Roodt, A.P., Brühl, C.A. (2021). Interspecific sensitivity of European amphibians towards two pesticides and comparison to standard test species. Environmental Sciences Europe 33:49. DOI: 10.1186/s12302-021-00491-1

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. DOI: 10.1016/j.scitotenv.2021.147865

Adams, E., Gerstle, V., Brühl, C.A. (2021). Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*). Environmental Toxicolology and Chemistry 50(5), 1289-1297. DOI: 10.1002/etc.4972

Adams, E., Leeb, C., Brühl, C.A. (2021). Pesticide exposure affects reproductive capacity of common toads (*Bufo bufo*) in a viticultural landscape. Ecotoxicology 30, 213-223. DOI: 0.1007/s10646-020-02335-9

Further scientific publications

Leeb, C., Schuler, L., **Adams, E.**, Brühl, C.A., Theissinger, K. (revised and resubmitted). Increased temperatures lead to lower toxicity of the fungicide folpet to larval stages of *Rana temporaria* and *Bufotes viridis.*

Adams, E., Brühl, C.A. (2020). Fungicide Exposure Induces Sensitivity Differences in Aquatic Life Stages of European Common Frogs (*Rana temporaria*). Journal of Herpetology 54(3), 331-336. DOI: 10.1670/19-004

Leeb, C., Kolbenschlag, S., Laubscher, A., **Adams, E.**, Brühl, C.A. (2020). Avoidance behavior of juvenile common toads (*Bufo bufo*) in response to surface contamination by different pesticides. PLoS ONE 15(11): e0242720. DOI: 10.1371/journal.pone.0242720

Lüderwald, S., Dackermann, V., Seitz, F., **Adams, E.**, Feckler, A., Schilde, C., Schulz, R., Bundschuh, M. (2019). A blessing in disguise? Natural organic matter reduces the UV light-induced toxicity of nanoparticulate titanium dioxide. Science of the Total Environment 663, 518-526. DOI: 10.1016/j.scitotenv.2019.01.282

Contributions to scientific conferences

2021

Adams, E., Leeb, C., Roodt, A.P., Brühl, C.A. (2021). Interspecies Sensitivity of Aquatic Life Stages of European Amphibians to Two Pesticides. Oral presentation. Virtual Young Environmental Scientists Meeting 2021

2020

Adams, E., Brühl, C.A. (2020). Reproductive health of common toads (*Bufo bufo*) in viticultural landscapes. Poster presentation. SETAC SciCon Europe 2020.

2019

Adams, E., Gerstle, V., Brühl, C.A. (2019). Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile *Rana temporaria*. Oral presentation. SETAC North America 2019, Toronto, Canada.

Adams, E., Gerstle, V., Brühl, C.A. (2019). Acute and chronic toxicity of environmentally relevant pesticide concentrations to early developmental stages of European Anura. Oral presentation. Young Environmental Scientists Meeting 2019, Ghent, Belgium.

2018

Adams, E., Leeb, C., Gerstle, V., Brühl, C.A. *et al.* (2018). Effects of pesticides on the development of two European amphibian species: a long-term semi-field study. Poster presentation. Gesellschaft für Ökologie 2018, Wien, Austria.

2017

Adams, E., Leeb, C., Brühl, C.A. (2017). Effects of the viticultural fungicide folpet (Folpan 80 WDG) on early developmental stages of Rana temporaria. Poster presentation. Young Environmental Scientists Meeting 2017, Stockholm, Sweden.

Adams, E., Leeb, C., Brühl, C.A. (2017). Effects of the viticultural fungicide folpet (Folpan 80 WDG) on early developmental stages of Rana temporaria. Poster presentation. SETAC GLB 2017, Neustadt an der Weinstraße, Germany.

Adams, E., Hauck, O., Schrimpf, A., Schulz, R., Slater, M.J. (2017). Maßnahmenkatalog für erfolgreiche, nachhaltige Besatzmaßnahmen autochthoner Edelkrebspopulationen. Oral presentation. Internationale Tagung des Forum Flusskrebse 2017, Vaduz, Liechtenstein.

Reports

Hauck, O., Slater, M.J., Schrimpf, A., Adams, E. (2018). Zwischenbericht des Alfred-Wegener-Instituts Helmholtz-Zentrum für Polar- und Meeresforschung (AWI) zum Projekt Maßnahmenkatalog für erfolgreiche, nachhaltige Besatzmaßnahmen autochthoner Edelkrebspopulationen (MaNaKa)