Amphibienschutz und Pestizideinsätze

Vom Fachbereich VI (Raum- und Umweltwissenschaften) der Universität Trier zur Erlangung des akademischen Grades Doktor der Naturwissenschaften (Dr. rer. nat.) genehmigte Dissertation

vorgelegt von

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Holotypus von *Xenopus laevis*. Aus: DAUDIN, F.-M. (1802): *An. XI. Histoire Naturelle des Rainettes, des Grenouilles et des Crapauds. Quarto version*. – Levraut (Paris).

Inhaltsverzeichnis

Verwendete Abkürzungen

ABl. EG	Amtsblatt der Europäischen Gemeinschaft
ABl. EU	Amtsblatt der Europäischen Union
Abs.	Absatz
AMPA	AminoMethylPhosphonic Acid
ANOVA	ANalysis Of VAriance
Art.	Artikel
ASTM	American Society for Testing and Materials
BGBl.	Bundesgesetzblatt
BfN	Bundesamt für Naturschutz
BMU	Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit
BNatSchG	Bundesnaturschutzgesetz
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
bzw.	beziehungsweise
cf.	vergleiche (aus dem Lateinischen confer)
DFG	Deutsche Forschungsgemeinschaft
EC	Effective Concentration (Wirkstoffkonzentration, welche bei einem bestimmten Prozentsatz der Versuchsorganismen innerhalb einer bestimmten Zeit einen definierten Effekt bewirkt)
EG	Europäische Gemeinschaft
e.g.	exempli gratia (Latein: zum Beispiel)
EU	Europäische Union
EWG	Europäische Wirtschaftsgemeinschaft
et al.	et alii / et aliae (Latein: und andere)
etc.	et cetera (Latein: und so weiter)
f.	und die folgende Dokumentseite
FETAX	Frog Embryo Teratogenesis Assay – *Xenopus*

ff.	und die folgenden Dokumentseiten
FFH	Flora-Fauna-Habitat
Fn.	Fußnote
GBl.	Gesetzblatt
ggf.	gegebenfalls
GR	Gewässerrandstreifen
GVBl.	Gesetz- und Verordnungsblatt
HPLC	High Performance Liquid Chromatography (Hochleistungsflüssigkeitschromatographie)
i.e.	id est (Latein: das heißt, das ist)
IUCN	International Union for Conservation of Nature
IUTR	Institut für Umwelt- und Technikrecht der Universität Trier
ISI	Institute for Scientific Information
i.V.m.	in Verbindung mit
kl.	Klepton (aus dem Griechischen klepto = ich stehle)
LC	Lethal Concentration (Wirkstoffkonzentration, welche bei einem bestimmten Prozentsatz der Versuchsorganismen innerhalb einer bestimmten Zeit letal wirkt)
lit.	littera (Lateini: Buchstabe)
m	Meter
mg/L	Milligramm pro Liter
N	Elementsymbol für Stickstoff
NAP	Nationaler Aktionsplan zur nachhaltigen Anwendung von Pflanzenschutzmitteln
n.g.	nicht genannt
NH_{4+}	Formel für Ammonium
NO_{3-}	Formel für Nitrat
NOEC	No Observed Effect Concentration (höchste Wirkstoffkonzentration, die keine messbare Wirkung verursacht)

Nr.	Nummer
OECD	Organisation for Economic Cooperation and Development (Organisation für wirtschaftliche Zusammenarbeit und Entwicklung)
P	Elementsymbol für Phosphor
PO_4^{3-}	Formel für Phosphat
POEA	POlyEthoxylated tallow Amine
PSM	Pflanzenschutzmittel
Rdn.	Randnotiz
S.	Seite(n)
SAC	Special Areas of Conservation
Tab.	Tabelle
TC	Teratogenic Concentration (Wirkstoffkonzentration, welche bei einem bestimmten Prozentsatz der Versuchsorganismen innerhalb einer bestimmten Zeit teratogene Effekte verursacht)
u.a.	unter anderem
USA	United States of America (Vereinigte Staaten von Amerika)
v.a.	vor allem
vgl.	vergleiche
WHG	Wasserhaushaltsgesetz
WRRL	Wasserrahmenrichtlinie
z.B.	zum Beispiel

Danksagungen

Mein besonderer Dank geht an PD Dr. STEFAN LÖTTERS für die Unterstützung und Förderung sowohl während als auch vor meiner Promotionsphase. Des Weiteren danke ich Prof. Dr. REINHARD HENDLER für die Betreuung des rechtswissenschaftlichen Teils meiner Doktorarbeit. Ohne die Expertise von PD Dr. BRUNO VIERTEL wären die amphibien-toxikologischen Laborversuche nicht möglich gewesen. Prof. Dr. MICHAEL VEITH trug ebenfalls – sowohl tatkräftig als auch durch seine Expertise – wesentlich zum Entstehen der vorliegenden Arbeit bei. Diese wurde dankenswerterweise durch das DFG-Graduiertenkolleg „VERBESSERUNG VON NORMSETZUNG UND NORMANWENDUNG IM INTEGRIERTEN UMWELTSCHUTZ DURCH RECHTS- UND NATURWISSENSCHAFTLICHE KOOPERATION" am INSTITUT FÜR UMWELT- UND TECHNIKRECHT der Universität Trier (IUTR) finanziert. Zudem geht mein Dank an das BUNDESAMT FÜR NATURSCHUTZ (BfN) für dessen Unterstützung.

Des Weiteren halfen folgende Personen (in alphabetischer Reihenfolge) bei den Freiland- und Laborarbeiten: LISA GASPER, MAX KERSCHBAUM, VALENTIN MINGO, RAINER RUFF, THERESA SCHEUREN und MAX SCHNEIDER.

Für die angenehme Zeit danke ich der gesamten Arbeitsgruppe der Biogeographie sowie dem Graduiertenkolleg.

Nicht zuletzt geht mein Dank natürlich an meine Familie, insbesondere an meine Eltern, die mich immer unterstützt hat.

Einleitende Worte

Diese Doktorarbeit wurde vom DFG-Graduiertenkolleg „VERBESSERUNG VON NORMSETZUNG UND NORMANWENDUNG IM INTEGRIERTEN UMWELTSCHUTZ DURCH RECHTS- UND NATURWISSENSCHAFTLICHE KOOPERATION“ des INSTITUTES FÜR UMWELT- UND TECHNIKRECHT der Universität Trier (IUTR) finanziert und im Fach Biogeographie der Universität Trier angefertigt. Die Arbeit behandelt – wie der Titel bereits nahelegt – das Themenfeld des Einflusses von Pestizidapplikationen auf die Amphibienfauna. Sie gliedert sich in zwei Hauptteile: zum einen besteht sie aus einem naturwissenschaftlichen Teil, welcher eine Sammeldisseration von vier bereits veröffentlichten und drei bisher unpublizierten Fachartikeln darstellt. Zum anderen besteht die Arbeit aus einem monographischen rechtswissenschaftlichen Teil.

Der **naturwissenschaftliche Teil** gliedert sich in drei Kapitel. Im **ersten Kapitel** werden zwei Fachartikel präsentiert, in denen das Risiko von Pestizideinsätzen auf Amphibien mit zwei unterschiedlichen methodischen Ansätzen bewertet wurde. Eine Meta-Analyse mit den Ergebnissen aus amphibientoxikologischen Studien zu dem am weltweit am häufigsten verwendeten Herbizidwirkstoff Glyphosat und seinen Formulierungen findet sich in:

WAGNER, N., REICHENBECHER, W., TEICHMANN, H., TAPPESER, B. & LÖTTERS, S. (2013): Questions concerning the potential impact of glyphosate-based herbicides on amphibians. – *Environmental Toxicology and Chemistry* **32**: 1688-1700. DOI: 10.1002/etc.2268

Ein GIS-basierter Ansatz zur Bewertung des Expositionsrisikos von Amphibienarten des FFH-Anhangs II gegenüber Pestiziden in ihren ausgewiesenen Schutzgebieten des Natura 2000-Netzwerkes fand Anwendung in:

WAGNER, N., RÖDDER, D., VEITH, M., BRÜHL, C.A., LENHARDT, P.P. & LÖTTERS, S. (2014): Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive. – *Biological Conservation* **176**: 64-70. DOI: 10.1016/j.biocon.2014.05.014

Das **zweite Kapitel** behandelt die durchgeführten amphibientoxikologischen Laborstudien mit zwei Anuren-Modellorganismen: dem Afrikanischen Krallenfrosch (*Xenopus laevis*) und dem Marokkanischen Scheibenzüngler (*Discoglossus scovazzi*). Diese beinhalten einerseits Versuche zu akuttoxischen Effekten eines ausgewählten Herbizids auf Embryonen und frühe Larven als auch die Effekte subletaler Konzentrationen auf unterschiedliche Entwicklungsstadien und die Metamorphose. Die Ergebnisse der Experimente lagen zur Abgabe der Dissertation noch als unpublizierte Manuskripte vor entweder, befanden sich jedoch teils bereits im „Peer-Review-Prozess“:

WAGNER, N., LÖTTERS, S., VEITH, M. & VIERTEL, B. (unpublished manuscript): Acute toxic effects of the herbicide formulation used in cycloxydim-tolerant maize cultivation on embryos and larvae of the African clawed frog, *Xenopus laevis*. – *Bulletin of Environmental Contamination and Toxicology* (revised version).

WAGNER, N., LÖTTERS, S., VEITH, M. & VIERTEL, B. (unpublished manuscript): Acute toxic effects of the herbicide formulation Focus® Ultra on embryos and larvae of the Moroccan painted frog, *Discoglossus scovazzi*. – *Archives of Environmental Contamination and Toxicology* (under review).

WAGNER, N., LÖTTERS, S., VEITH, M. & VIERTEL, B. (unpublished manuscript): No developmental effects but time-lag mortality of environmentally relevant Focus® Ultra concentrations on *Xenopus laevis* larvae.

Das **dritte Kapitel** fasst zwei Freilandarbeiten zusammen. In einer freilandökologischen Arbeit wurde untersucht, ob sich Larven und Metamorphlinge einer häufigen, einheimischen Anurenart (des Grasfrosches, *Rana temporaria*) als Bioindikatoren für die Kontamination von Kleingewässern mit Agrochemikalien eignen:

WAGNER, N., ZÜGHART, W., MINGO, V. & LÖTTERS, S. (2014): Are deformation rates of anuran developmental stages suitable indicators for environmental pollution? Possibilities and limitations. – *Ecological Indicators* **45**: 394-401. DOI: 10.1016/j.ecolind.2014.04.039

In einem Verhaltensexperiment wurde getestet, ob drei einheimische Amphibienarten (*Rana temporaria, Ichthyosaura alpestris, Lissotriton helveticus*) Gewässer meiden, welche mit umweltrelevanten Konzentrationen unterschiedlicher Stoffe kontaminiert wurden:

WAGNER, N. & LÖTTERS, S. (2013): Effects of water contamination on site selection by amphibians: experiences from an arena approach with European frogs and newts. – *Archives of Environmental Contamination and Toxicology* **65**: 98-104. DOI: 10.1007/s00244-013-9873-9

Der **rechtswissenschaftliche** Teil beschäftigt sich mit den Aspekten des Schutzes von Kleingewässern – Reproduktionsstätten der meisten einheimischen Amphibien – durch Gewässerrandstreifen vor schädlichen Stoffeinträgen und geht detailliert auf die europarechtlichen und nationalen Rechtsgrundlagen ein.

Im Rahmen der durchgeführten naturwissenschaftlichen Arbeiten halfen verschiedene Personen bei der Konzepterstellung (Tabelle 1), Prof. Dr. REINHARD HENDLER bei der des rechtswissenschaftlichen Teils. Bei der Aufnahme und Auswertung der Daten halfen v.a. Personen, welche auch Teile der gewonnenen Daten für ihre Abschlussarbeiten nutzten. Schließlich trugen die jeweiligen Ko-Autoren der Artikel (in unterschiedlichem Maße) bei der Manuskripterstellung bei. Prof. Dr. REINHARD HENDLER half beim Verfassen des rechtswissenschaftlichen Teils der vorliegenden Arbeit. Die Tabelle 1 stellt eine Übersicht des Arbeitsanteiles dar, welchen ich selbst zum Entstehen beigetragen habe.

Tab. 1: Eigener Arbeitsanteil (%) zum Entstehen der Fachartikel, welche in den naturwissenschaftlichen Teil der vorliegenden Doktorarbeit einflossen.

Beteiligte Personen sind in Initalen gegeben (alphabetisch nach Familiennamen geordnet): Carsten Brühl (CB), Lisa Gasper (LG), Max Kerschbaum (MK), Patrick Lenhardt (PL), Stefan Lötters (SL), Valentin Mingo (VM), Rainer Ruff (RR), Wolfram Reichenbecher (WR), Dennis Rödder (DR), Theresa Scheuren (TS), Max Schneider (MS), Beatrix Tappeser (BT), Hanka Teichmann (HT), Michael Veith (MV), Bruno Viertel (BV), Wiebke Züghart (WZ).

Artikel	**Konzept-erstellung**	**Daten-aufnahme**	**Statistische Auswertung**	**Verfassen des Manuskriptes**
„Questions concerning the potential impact of glyphosate-based herbicides on amphibians”	75% SL	100%	100%	60% SL, WR, HT, BT
„Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive”	75% SL, DR, CB	80% MV	100%	75% SL, DR, CB, MV, PL
„Acute toxic effects of the herbicide formulation used in cycloxydim-tolerant maize cultivation on embryos and larvae of the African clawed frog, *Xenopus laevis*“	60% BV	90% MS, MV	100%	60% BV, MV, SL
„Acute toxic effects of the herbicide formulation Focus® Ultra on embryos and larvae of the Moroccan painted frog, *Discoglossus scovazzi*”	60% BV	90% MV	100%	60% BV, MV, SL
„No developmental effects but time-lag mortality of environmentally relevant Focus® Ultra concentrations on *Xenopus laevis* larvae. “	50% BV	50% LG, MK, MV	100%	60% BV, MV, SL
„Are deformation rates of anuran developmental stages suitable indicators for environmental pollution? Possibilities and limitations”	75% SL	50% VM	100%	80% SL, WZ, VM
„Effects of water contamination on site selection by amphibians: experiences from an arena approach with European frogs and newts”	60% SL	50% TS, RR	100%	80% SL

In meiner Promotionszeit an der Universität Trier wurden – neben den in dieser Schrift zusammengefassten – weitere dreizehn begutachtete Artikel in Fachzeitschriften (davon sieben ISI-gelistete), zwei begutachtete Buchkapitel und ein BfN-Skript verfasst und veröffentlicht (ein Artikel befand sich zur Abgabe der Dissertation noch im Druck), welche sich hauptsächlich herpetologischen, arten- und naturschutzfachlichen sowie umweltrechtlichen Fragestellungen widmen (siehe nachfolgende Aufzählung). Für das Graduiertenkolleg besonders hervorhebenswert erachte ich hierbei die interdisziplinäre Publikation *„Abbaugebiete als Sekundärlebensraum streng geschützter Amphibienarten – Rekultivierung im Licht des europäischen Artenschutzrechtes“*, welche ich zusammen mit Jenny Kirschey (Kollegiatin des Graduiertenkollegs) angefertigt habe sowie die interdisziplinäre Publikation *„Europe needs a new vision for a Natura 2020 network“*, welche durch mehrere Doktoranden/innen und Dozenten des Graduiertenkollegs verfasst wurde. Beide Werke verdeutlichen die gute Zusammenarbeit zwischen Natur- und Rechtswissenschaftlern am Trierer Graduiertenkolleg.

ISI-gelistete Artikel:

1. Böll, S., Schmidt, B.R., Veith, M., **Wagner, N**., Rödder, D., Weimann, C., Kirschey, T. & Lötters, S. (2013): Anuran amphibians as indicators for changes in aquatic and terrestrial ecosystems following GM crop cultivation: a monitoring guideline. – *BioRisk* **8**: 39-51. DOI: 10.3897/biorisk.8.3251
2. Chiari, Y., van der Meijden, A., Mucedda, M., **Wagner, N.** & Veith, M. (2013): No detection of the pathogen *Batrachochytrium dendrobatidis* in Sardinian cave salamanders, genus *Hydromantes* – *Amphibia-Reptilia* **34**: 136-141. DOI: 10.1163/15685381-00002876
3. Göçmen, B., Veith, M., Iğci, N., Akman, B. Godmann, O. & **Wagner, N.** (2013): No detection of the amphibian pathogen *Batrachochytrium dendrobatidis* in terrestrial Turkish salamanders (*Lyciasalamandra*) despite its occurrence in syntopic frogs (*Pelophylax bedriagae*). – *Salamandra* **49**: 51-55.
4. Hochkirch, A., Schmitt, T., Beninde, J., Hiery, M., Kinitz, T., Kirschey, J., Matenaar, D., Rohde, K., Stoefen, A., **Wagner, N.**, Zink, A., Lötters, S., Veith, M. & Proelß, A. (2013): Europe needs a new vision for a Natura 2020 network. – *Conservation Letters* **6**: 462–467. DOI: 10.1111/conl.12006
5. Hochkirch, A., Schmitt, T., Beninde, J., Hiery, M., Kinitz, T., Kirschey, J., Matenaar, D., Rohde, K., Stoefen, A., **Wagner, N.**, Zink, A., Lötters, S., Veith, M.

& PROELß, A. (2013): How much biodiversity does Natura 2000 cover? – *Conservation Letters* **6**: 470–471. DOI: 10.1111/conl.12037

6. LÖTTERS, S., KIELGAST, J., SZTATECSNY, M., **WAGNER, N.**, SCHULTE, U., WERNER, P., RÖDDER, D., DAMBACH, J., REISSNER, T., HOCHKIRCH, A. & SCHMIDT, B.R. (2012): Absence of infection with the amphibian chytrid fungus in the terrestrial Alpine salamander, *Salamandra atra*. – *Salamandra* **48**: 58-62. DOI: 10.5167/uzh-62111
7. LÖTTERS, S., FILZ, K.J., **WAGNER, N.**, SCHMIDT, B.R., EMMERLING, C. & VEITH, M. (im Druck): Hypothesizing if responses to climate change affect herbicide exposure risk for amphibians. – *Environmental Sciences Europe*.

Sonstige begutachtete Artikel:

8. BENINDE, J., HAENSCH, M., HIERY, M., KINITZ, T., KIRSCHEY, J., MATENAAR, D., ROHDE, K., STOEFEN, A., **WAGNER, N.** & ZINK, A. (2013): Workshop-Bericht: „Anthropogene Störungen mariner Ökosysteme in Deutschland – eine natur- und rechtswissenschaftliche Bewertung“. – *Natur und Recht* **35**: 410-412. DOI: 10.1007/s10357-013-2461-y
9. KIRSCHEY, J. & **WAGNER, N.** (2013): Abbaugebiete als Sekundärlebensraum streng geschützter Amphibienarten – Rekultivierung im Licht des europäischen Artenschutzrechtes. – *Zeitschrift für Europäisches Umwelt- und Planungsrecht* **4**: 282-289.
10. SCHULTE, U., KIRCHHOF, S. & **WAGNER, N.** (2012): Populationsgröße, Abundanzen und Habitatnutzung einer Schlingnatter-Population (*Coronella austriaca*) bei Trier. – *Zeitschrift für Feldherpetologie* **19**: 185-200.
11. SCHULTE, U., HOCHKIRCH, A., **WAGNER, N**. & JACOBY, P. (2013): Witterungsbedingte Antreffwahrscheinlichkeit der Schlingnatter (*Coronella austriaca*). – *Zeitschrift für Feldherpetologie* **20**: 197-209
12. THIESMEIER, B. KORDGES, T. & **WAGNER, N.** (2013): Phänologie und Morphometrie einer Blindschleichen-Population (*Anguis fragilis*) in Hattingen (NRW). – *Zeitschrift für Feldherpetologie* **20**: 65-78.
13. **WAGNER, N.** (2012): Occupation of Monk Parakeet (*Myiopsitta monachus*) nest cavities by introduced House Sparrows (*Passer domesticus*) in Rio Grande do Sul, Brazil. – *Boletín de la Sociedad Antioqueña de Ornitología* **20**: 72-78.

Begutachtetes BfN-Skript und Buchkapitel:

14. **WAGNER, N.** & LÖTTERS, S. (2013): *Possible correlation of the worldwide amphibian decline and the increasing use of glyphosate in the agrarian industry.* – BfN-Skripten **343**, Bundesamt für Naturschutz, Bonn, 202 pp.
15. TRAUTMANN, S., LÖTTERS, S., OTT, J., BUSE, J., FILZ, K., RÖDDER, D., **WAGNER, N.**, JAESCHKE, A., SCHULTE, U., VEITH, M., GRIEBELER, E.-M., & BÖHNING-GAESE, K. (2012): Auswirkungen auf geschützte und schutzwürdige Arten. In: MOSBRUGGER, V., BRASSEUR, G., SCHALLER, M. & STRIBRNY, B. (eds.): *Klimawandel und Biodiversität – Folgen für Deutschland.* – WBG Press, Darmstadt: 260-289.
16. **WAGNER, N**., REICHENBECHER, W., TEICHMANN, B., TAPPESER, B. & LÖTTERS, S. (2013): Are frogs and toads affected by complementary herbicides of GM crops? In: BRECKLING, B. & VERHOEVEN, R. (eds.): *GM-Crop Cultivation – Ecological Effects on a Landscape Scale – Proceedings of the Third GMLS-Conference 2012 in Bremen – Theorie in der Ökologie 17* – Peter Lang, Frankfurt: 125-129.

1. Naturwissenschaftlicher Teil

Einleitung

Weltweite Populationsrückgänge und Artensterben bei Amphibien

Weltweit stellen Amphibien die am stärksten gefährdete Wirbeltierklasse dar und etwa ein Drittel der derzeit bekannten rund 7000 beschriebenen Arten ist vom Aussterben bedroht (ALFORD & RICHARDS 1999; STUART *et al.* 2004, 2008; WAKE & VREDENBURG 2008). Obwohl in manchen Regionen – wie etwa in vielen Industrieländern – die stärksten Populationsrückgänge schon längere Zeit zurückliegen (HOULAHAN *et al.* 2000), wurden die beobachteten unnatürlich hohen Bestandsrückgänge und Extinktionen erst 1989 beim ersten herpetologischen Weltkongress als globales Muster diskutiert (COLLINS & STORFER 2003). Über die Ursachen wird seither intensiv geforscht, auch über die Frage, welche Populationsrückgänge und Aussterbeereignisse tatsächlich unnatürlich sind (MENDELSON *et al.* 2006; LIPS *et al.* 2006; GASCON *et al.* 2007). Doch ist es sehr wahrscheinlich, dass der Großteil der Ursachen anthropogen bedingt ist (PECHMANN *et al.* 1991; MCCOY 1994; COLLINS & STORFER 2003; GASCON *et al.* 2007; WAKE & VREDENBURG 2008). In den letzten Jahrzehnten sind katastrophale Rückgänge und Aussterbeereignisse aus montanen, oftmals abgelegenen Regionen in Amerika, Australien und Südeuropa zu verzeichnen (BERGER *et al.* 1998; LIPS 1998; LA MARCA *et al.* 2005; BOSCH & MARTINEZ-SOLANO 2006; LIPS *et al.* 2006). Aufgrund der Abgeschiedenheit der Gebiete werden direkte anthropogene Einflüsse, wie etwa Lebensraumzerstörung, hier eher als zweitrangig betrachtet und die sich ausbreitende Hautkrankheit Chytridiomykose, ausgelöst durch den Chytridpilz *Batrachochytrium dendrobatidis*, ist in diesen Gebieten wohl die Hauptursache (LIPS *et al.* 2006). Umgekehrt wird davon ausgegangen, dass der Pilz in viele Teile der Welt durch den Menschen eingeführt wurde und die dortigen Amphibienpopulationen daher stark negativ beeinflusst werden, weil sie nicht evolutiv an den Parasiten angepasst sind (FISHER *et al.* 2009). Vor kurzer Zeit wurde in Europa zudem eine weitere Chytridpilzart neu beschrieben, welche auf Schwanzlurche spezialisiert ist und bei diesen Massensterben verursachen kann (MARTEL *et al.* 2013). Auch hier wird vermutet, dass der Pilz ursprünglich in Asien beheimatet ist und erst durch den Tierhandel nach Europa eingeführt wurde (MARTEL *et al.* 2014). Das Beispiel der Chytridiomykose zeigt, dass ein Faktor selten alleine wirkt, sondern meist mit anderen zusammen (hier: parasitäre Krankheit + Globalisierung). COLLINS & STORFER (2003) ordneten sechs Haupthypothesen, welche für das weltweite Amphibiensterben verantwortlich gemacht werden, in zwei Klassen. Die erste Klasse beinhaltet Faktoren, welche Amphibien bereits seit über einem Jahrhundert negativ beeinflussen (Landnutzungswandel, der Habitate zerstört; Neozoen, welche einheimischen Arten schaden; übermäßige Ausbeutung von Amphibien zu Nutzungszwecken). In der zweiten Klasse werden Ursachen geführt, die eher rezenter Natur sind und am stärksten seit etwa Mitte der 1990er Jahre wirken („*global change*" inklusive erhöhter UV-Einstrahlung und Klimawandel; neu auftretende Infektionskrankheiten, besonders der Amphibien-Chytridpilz; Umweltverschmutzung). Insgesamt scheinen auch nach diesen Autoren komplexe Interaktionen verschiedener Faktoren am Werke zu sein (COLLINS & STORFER 2003). Ein in der zweiten Klasse geführter, neuerer Faktor ist die Umweltverschmutzung, wobei hier explizit auch der vermehrte Einsatz von Agrochemikalien genannt wird (COLLINS & STORFER 2003; HAYES *et al.* 2006; BOONE *et al.* 2007).

Vorkommen von Amphibien in landwirtschaftlich genutzten Gebieten

Obwohl landwirtschaftlich genutzte Landschaften stark anthropogen verändert sind, beherbergen sie noch viele Amphibienpopulationen, welche meistens in landwirtschaftlich genutzten Regionen persistieren (MANN *et al.* 2009; BERGER *et al.* 2011) – im Gegensatz zu vielen anderen Offenlandarten (Pflanzen, Vögel), welche oftmals nur wegen der landwirtschaftlichen Nutzung in einem Gebiet vorkommen. Besonders Amphibienarten des Tieflands persistieren in der Kuturlandschaft, da hauptsächlich ihre Lebensräume für die landwirtschaftliche Nutzung umgestaltet wurden und werden (GALLANT *et al.* 2007). Adulte und juvenile Amphibien finden etwa in extensiv genutzten Randstrukturen, etwa in seltener gemähten Wiesen oder unter Feldgehölzen, terrestrischen Lebensraum, kommen jedoch auch direkt auf der landwirtschaftlich genutzten Fläche vor, wo sie etwa nach Nahrung suchen (BERGER *et al.* 2011; WAGNER & LÖTTERS 2013). Zudem müssen viele Arten, welche jährliche Wanderungen vollziehen, landwirtschaftliche Nutzflächen überqueren (BERGER *et al.* 2011, 2012, 2013). Kleine Tümpel, Entwässerungsgräben und andere Kleinstgewässer werden zu Reproduktionszwecken angenommen (KNUTSON *et al.* 2004). Selbst temporär überflutete Wiesen und Felder als auch mit Wasser gefüllte Wagenspuren werden von manchen Arten als Laichgewässer genutzt (WAGNER & LÖTTERS 2013). Beachtet man, dass etwa die Hälfte der deutschen Landesfläche landwirtschaftlich genutzt wird (STATISTISCHES

BUNDESAMT 2012), ist es wenig verwunderlich, dass es bezüglich des Amphibienschutzes Bedenken zu den negativen Auswirkungen der Landwirtschaft gibt. Neben der direkten Zerstörung von Primärhabitaten wie Auen, welche auch für die Landwirtschaft umgestaltet wurden, diese aber meist schon längere Zeit zurückliegt (KIRSCHEY & WAGNER 2013), beeinträchtigt die fortschreitende Intensivierung der Landwirtschaft, etwa durch weiteres Zusammenlegen von Feldern, Grünlandumbruch und Wegfall extensiv genutzter Saumstrukturen, das Fortbestehen von Amphibienpopulationen in der Kulturlandschaft (WAGNER & LÖTTERS 2013). Mahd (LICZNER 1999), Pflügen und andere mechanische Bodenbearbeitung (DÜRR *et al.* 1999) können je nach eingesetztem Gerät zu bedeutenden Verlusten bei den terrestrischen Lebensstadien von Amphibien führen. Ebenso kann das Entfernen von Laichgewässern ausschlaggebend für das Erlöschen von Amphibienpopulationen in der Agrarlandschaft sein (CURADO *et al.* 2011). Wirtschaftsdünger (Gülle, Jauche) und Klärschlamm können durch landwirtschaftliche Tätigkeit in Gewässer gelangen, hohe Phosphat- und Stickstoffkonzentrationen zudem standortspezifisch auch bereits als natürliche Grundlast vorliegen (LINDEN 1993; WAGNER *et al.* 2014). Eine anthropogene Erhöhung von Phosphat- und Stickstoffkonzentration kann besonders in Kleingewässern zu deren (unnatürlichen) Eutrophierung führen, was zuerst sogar positive Effekte für Amphibienlarven (nicht jedoch für andere Taxa) mit sich bringen kann, indem das Algenwachstum gefördert wird und somit mehr Versteckmöglichkeiten vorhanden sind und (für herbivore Anurenlarven) die verfügbare Nahrungsmenge erhöht wird (MANN *et al.* 2009; EARL & WHITEMAN 2010). Im weiteren Verlauf können jedoch anoxische Verhältnisse entstehen, was zu einem Absterben der Larven führt (MANN *et al.* 2009). Durch erhöhtes Algenwachstum vermehren sich auch bestimmte Wasserschnecken verstärkt, welche parasitären Trematoden als Zwischenwirt dienen. Somit erhöht sich durch eine Eutrophierung der Laichgewässer das Infektionsrisiko von Anurenlarven mit diesen Trematoden, welche Missbildungen (besonders der Extremitäten) hervorrufen, welche letztendlich meist zum Tod der metamorphosierten Tiere führen (JOHNSON *et al.* 2007). Bisher wurde dieser indirekte negative Effekt eutropher Gewässer jedoch nur in Nordamerika beschrieben. Ein weiterer potenzieller Gefährdungsfaktor ist der Einsatz von Agrochemikalien, also Handelsdünger und Pestizide, wobei sich in der vorliegenden Dissertationsschrift detailliert auf den Einfluss von Pestiziden auf Amphibien bezogen werden soll. Zusammenfassend wirkt die moderne landwirtschaftliche Nutzung also mehrfach negativ auf Amphibien. Bezieht man sich wiederum auf die von COLLINS & STORFER (2003) aufgestellten Klassen, ist die Landwirtschaft für zwei Faktoren mitverantwortlich, welche bereits seit über einem

Jahrhundert (Landnutzungswandel) als auch eher rezent (Umweltverschmutzung) negativ auf Amphibienpopualtionen wirken (Abb. 1).

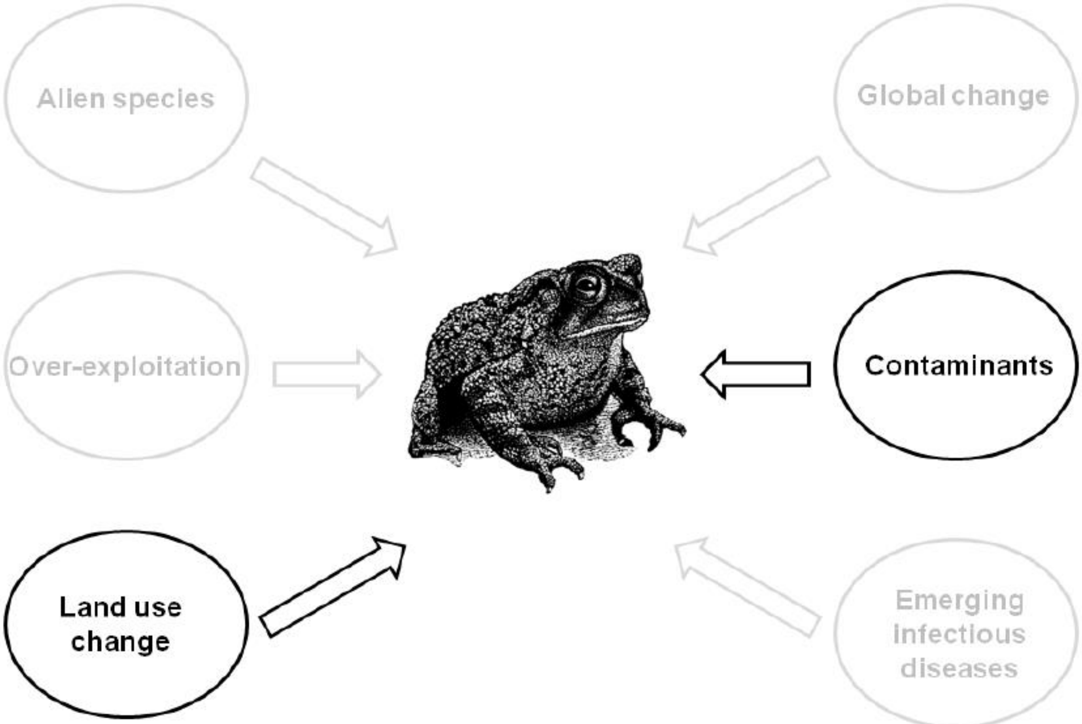

Abb. 1: Mitverantwortlickeit der Landwirtschaft zu zwei von den von COLLINS & STORFER (2003) aufgestellten sechs Haupthypothesen für das weltweite Amphibiensterben (Abbildung aus WAGNER & LÖTTERS 2013).

Expositionspfade gegenüber Pestiziden

Amphibien, die bei saisonalen Wanderungen Felder überqueren, auf ihnen rasten oder Nahrung suchen, können direkt durch Applikationen, besonders direktes Übersprühen, geschädigt werden (BERGER *et al.* 2013; BRÜHL *et al.* 2013). BRÜHL *et al.* (2013) wiesen nach, dass bis zu 100% juveniler Grasfrösche (*Rana temporaria*) nach Exposition gegenüber empfohlener Applikationsmengen bestimmter, in Deutschland häufig verwendeter, Fungizide starben. Ebenso ist indirekte Kontamination über die Nahrung, wie übersprühte Arthropoden, oder Kontakt mit kontaminierten Boden oder Pflanzenmaterial möglich (BAKER 1985; MCCOMB *et al.* 2008; BERGER *et al.* 2011; BRÜHL *et al.* 2011). Tiere können in ihren terrestrischen als auch aquatischen Habitaten zudem durch Abdrift, Drainage- und

Oberflächenabfluss gegenüber Pestiziden exponiert werden (BROWNER 1994; DAVIDSON *et al.* 2002; DAVIDSON 2004; DAVIDSON & KNAPP 2007; BROWN & VAN BEINUM 2009).

Effekte von Pestiziden auf Amphibien

Kausale Zusammenhänge zwischen Pestizideinsätzen und deren Effekte auf Amphibienpopulationen sind noch nicht eindeutig geklärt (SCHMIDT 2004; MANN *et al.* 2009; WAGNER *et al.* 2013). Dies liegt zum einen daran, dass verglichen zu anderen Tieren der Agrarlandschaft, wie etwa Brutvögeln, für Amphibien relativ wenig Daten zu Verbreitung und Abundanzen vorliegen und dass, was bei etwa Tausend zugelassener und potenziell eingesetzter Mittel (BVL 2014) weniger verwunderlich ist, meist Daten zu tatsächlicher Lebensraumkontamination und Exposition fehlen (WAGNER *et al.* 2013). Jedoch hat der steigende Einsatz von Agrochemikalien eindeutig das Potenzial – besonders im Zusammenspiel mit weiteren Stressoren – Amphibienpopulationen langfristig zu schädigen (MANN *et al.* 2009). Effekte sind jedoch fast immer nur auf der Individualebene dokumentiert. Obwohl einige Studien nahelegen, dass sich Amphibien aus dem Agrarland evolutiv an den Stress durch wiederholte Applikation bestimmter Pestizide angepasst haben und dadurch eine gewisse Resistenz erworben zu haben scheinen (COTHRAN *et al.* 2013; HUA *et al.* 2013), zeigen andere Studien eindeutig adverse Effekte des Pestizideinsatzes auf Individuen, welche in der Kulturlandschaft persisitieren. Bei Agakröten (*Rhinella marina*), welche in intensiv landwirtschaftlich genutzten Gebieten leben, konnte etwa eine erhöhte Ausbildung anormaler Gonaden beobachtet werden (MCCOY *et al.* 2008). Metamorphlinge aus solchen Gebieten besitzen oftmals eine verminderte Fitness, sind kleiner und leichter und die Metamorphose erfolgt früher verglichen mit Tieren aus natürlichen Gewässern, was sowohl in Südamerika (*Leptodactylus mystacinus*) als auch in Europa beim Grasfrosch (*Rana temporaria*) nachgewiesen werden konnte (ATTADEMO *et al.* 2014; WAGNER *et al.* 2014). Sowohl bei Adulti verschiedener südamerikanischer Anurenarten, welche in landwirtschaftlich genutzten Gebieten leben, als auch bei ihren Larven aus Gewässern, welche im Agrarland gelegen sind, konnte oxidativer Stress und Einflüsse auf die Aktivität von Enzymen belegt werden, welche bei der Detoxifikation und Neurotransmission beteiligt sind (ATTADEMO *et al.* 2007, 2014; LAJMANOVICH *et al.* 2010). Dies kann auch dazu führen, dass solch gestresste Tiere krankheitsanfälliger sind, d.h. subletale Pestizidkonzentrationen immunsuppressiv wirken (ATTADEMO *et al.* 2011). Interessanterweise konnten jedoch die wenigen, bisher durchgeführten Studien kein erhöhtes Infektionsrisikio mit dem Amphibien-Chytridpilz durch gleichzeitigen pestizidinduzierten Stress nachweisen (z.B. DAVIDSON *et al.* 2007; GAHL *et al.* 2011; PAETOW *et al.* 2012), jedoch dass etwa das Insektizid Carbaryl die angeborene Immunantwort stark schwächt (DAVIDSON *et al.* 2007). Außerdem wurden zumeist Arten getestet, welche für den Pilz weniger anfällig scheinen. Weitere indirekte Effekte von Pestizideinsätzen beinhalten etwa die Meidung kontaminierter Gewässer (TAKAHASHI 2007; VONESH & BUCK 2007; VONESH & KRAUS 2009). Meistens belegen aber keine Feldarbeiten, sondern Mesokosmos- und besonders Laborstudien adverse Effekte von Pestiziden auf Amphibien. Diese sind von besonderem naturschutzfachlichem Interesse, wenn umweltrelevante Pestizidkonzentrationen in den Versuchen Verwendung fanden (z.B. RELYEA 2009; WILLIAMS & SEMLITSCH 2010; BRÜHL *et al.* 2013). Es wurden akuttoxische (z.B. RELYEA 2005a; RELYEA & JONES 2009; BRÜHL *et al.* 2013) als auch chronische und verzögerte Effekte (z.B. HOWE *et al.* 2004; CAUBLE & WAGNER 2005; HAMMOND *et al.* 2012) auf Amphibien und ihre Larven nachgewiesen. Pestizide können endokrin wirken, etwa die Schilddrüsen-Achse beeinflussen, was sich auf den Zeitpunkt der Metamorphose auswirken kann (HOWE *et al.* 2004), was sich mit den Beobachtungen aus dem Freiland deckt. BAKER *et al.* (2013) konnten durch eine großangelegte Meta-Analyse der Ergebnisse publizierter Studien zu den Effekten von Agrochemikalien auf Amphibien nachweisen, dass Agrochemikalien eindeutig das Überleben und das Wachstum von Amphibien negativ beeinflussen, unabhängig von artspezifischen Unterschieden. Negative Einflüsse verschiedener chemischer Klassen auf Überleben oder Wachstum unterscheiden sich in ihrer Wahrscheinlichkeit, Organophosphate z.B. beeinflussen jedoch beide Parameter.

Die Effekte von Pestiziden auf Amphibien sind folglich vielfältig und es besteht besonders im Freiland noch viel Forschungsbedarf. In der vorliegenden Arbeit wurden daher wichtige Fragestellungen zu diesem Themengebiet aufgegriffen. Der potenzielle Einfluss von Pestizideinsätzen auf Amphibien, besonders wildlebende, stellt ein komplexes Arbeitsfeld dar, so dass verschiedene methodische Ansätze benötigt werden, um auf verschiedene Fragestellungen einzugehen. Daher ist der naturwissenschaftliche Teil der vorliegenden Dissertationsschrift in unterschiedliche Kapitel gegliedert, welche unterschiedliche Fragestellungen behandeln, an welche mit geeigneten Methoden herangegangen wurde.

Das **erste Kapitel** befasst sich mit unterschiedlichen Fragestellungen zur Risikobewertung des Einsatzes von bestimmten Pestiziden (glyphosatbasierten Herbiziden) auf Amphibien als auch des allgemeinen intensivierten Einsatzes von Agrochemikalien speziell in den Schutzgebieten europarechtlich geschützter Amphibienarten.

Der potenzielle Einfluss glyphosatbasierter Herbizide auf Amphibien

Herbizide mit dem aktiven Wirkstoff Glyphosat dominieren den Weltmarkt, besonders aufgrund ihrer Nutzung beim Anbau von Nutzpflanzen, welche durch gentechnische Veränderung eine hohe Toleranz gegenüber diesem Wirkstoff aufweisen (DILL *et al.* 2005, 2010; DUKE & POWLES 2008). Doch auch in Deutschland und den meisten anderen europäischen Ländern, wo sich die Nutzung der meisten gentechnisch veränderten Organismen noch in der Zulassung befindet und diese nur extrem kleinräumig – meist zu Forschungszwecken – angebaut werden (mit Ausnahmen wie Spanien, wo fast ein Drittel des Maisanbaus bereits mit gentechnisch modifizierten Pflanzen erfolgt: www.isaaa.org/), steigt der Absatz glyphosatbasierter Herbizide; hauptsächlich, weil sie zur allgemeinen Unkrautvernichtung vor Beginn des Anbaus verschiedener Nutzpflanzen Verwendung finden („*no tillage farming*“: RAUBUCH & SCHIEFERSTEIN 2002), jedoch etwa auch in der sogenannten Sikkation von Getreide – d.h. dem Abtöten der Getreidepflanze vor der Ernte durch Herbizideinsatz, so dass das Getreide an der Luft vor der Lagerung trocknen kann – bei der Unkrautkontrolle im Weinbau, an Verkehrswegen (insbesondere Bahngleisen), als auch im privaten Bereich (DILL *et al.* 2010). Wird ein Pestizid vermehrt in der Umwelt ausgebracht, erhöht sich damit auch das Risiko, dass Nichtzielorganismen wie Amphibien in Kontakt mit ihm kommen. BERGER *et al.* (2013) zeigten für ihr Untersuchungsgebiet, dass sich Anwendungen glyphosatbasierter Herbizide in der Landwirtschaft größtenteils mit den Wanderbewegungen der dortigen Amphibienpopualtionen decken. Ende des letzten Jahrtausends wurden erstmalig Effekte glyphosatbasierter Herbizide auf Amphibien untersucht (MANN & BIDWELL 1999). Da diese Autoren die Umweltsicherheit glyphosatbasierter Herbizide in Frage stellten, folgten weitere Arbeiten. Besonders die Studien von RICK A. RELYEA und dessen Kollegen fanden ein großes Medienecho. Von der Hauptherstellerfirma glyphosatbasierter Herbizide (als auch anderen Wissenschaftlern) wurden Methodik und Schlussfolgerungen kritisiert (v.a. zu RELYEA 2005b; siehe auch RELYEA 2006 vs. THOMPSON *et al.* 2006). Daraufhin stieg die Zahl wissenschaftlicher Studien zu den Effekten von Glyphosat und seiner Formulierungen auf Amphibien weiter an. Jedoch variierten die Studiendesigns sowie die Schlussfolgerungen der verschiedenen Arbeiten. Dies kann auch damit zusammenhängen, dass viele Studien zu den Effekten von Pestiziden auf

Nichtzielorganismen von der Herstellerfirma selbst oder aber durch diese finanziert zustande kommen, was einen starken Interessenkonflikt darstellt, auf den BOONE *et al.* (2014) zurecht hinweisen. Die (zu Beginn der Promotionsphase) publizierte Literatur zu den Effekten von Glyphosat und glyphosatbasierten Herbiziden auf Amphibien wurde mit Hilfe einer qualitativen Meta-Analyse objektiv bewertet. Diese Ergebnisse wurden in der Fachzeitschrift *„Environmental Toxicology and Chemistry"* publiziert, welche von der „*Society of Environmental Toxicology and Chemistry"* (SETAC) herausgegeben wird. Die Ergebnisse wurden auf einer internationalen Tagung präsentiert (*International Conference on Implications of GM Crop Cultivation at Large Spatial Scales (GMLS),* Bremen, 14.06.2012).

Die zugrunde liegenden Fragestellungen lauteten:

„Was ist der aktuelle Wissensstand zu Effekten von Glyphosat und glyphosatbasierten Herbiziden auf Amphibien?"

„Welche Endpunkte sind am besten geeignet, um potenzielle Effekte von glyphosatbasierten Herbiziden und des Wirkstoffes auf Amphibien nachzuweisen?"

Bewertung des Pestizid-Expositionsrisikos der Amphibienarten des Anhangs II der FFH-Richtlinie in ihren Schutzgebieten des Natura 2000-Netzwerkes

Das Natura 2000-Netzwerk der Europäischen Union, welches aus Schutzgebieten besteht, die nach den Maßgaben der FFH-Richtlinie und der europäischen Vogelschutzrichtlinie ausgewiesen wurden, wird als eines der größten und wichtigsten Schutzgebietsnetzwerke weltweit angesehen (LOCKWOOD 2006). Anhang II der FFH-Richtlinie listet Tier- und Pflanzenarten von „gemeinschaftlichem Interesse" für welche besondere Schutzgebiete („*Special Areas of Conservation*" = SAC) ausgewiesen werden müssen, um die Populationen dieser Arten zu schützen (EU 1992). Insgesamt werden 22 europäische Amphibienarten und drei Unterarten in Anhang II geführt; vier davon sind sogar sogenannte „prioriäre Arten" der FFH-Richtlinie, was einen erhöhten Schutzstatus bedeutet (EU 1992). In den SAC ist landwirtschaftliche Nutzung weiterhin möglich, solange sich diese nicht nachteilig auf die Lebensräume und darin vorkommenden Arten auswirkt (EU 1992). Durch eine landwirtschaftliche Nutzung können die Populationen der Anhang II-Amphibienarten jedoch potenziell in ihren Schutzgebieten gegenüber Pestiziden exponiert werden (siehe generelle Einleitung). Ob Pestizideinsätze in den SAC jedoch tatsächlich ein Verschlechterungsverbot darstellen, ist sehr schwer zu beantworten, da kausale Zusammenhänge zwischen dem Einsatz von Pestiziden und deren Auswirkungen auf Amphibienpopulationen schwer zu ziehen sind (SCHMIDT 2004; MANN *et al.* 2009; WAGNER *et al.* 2013; siehe auch den rechtswissenschaftlichen Teil der vorliegenden Arbeit). Daten zu tatsächlicher Habitatkontamination, Vorkommen von Arten in und angrenzend an landwirtschaftliche Nutzflächen sowie deren langjährige Bestandsentwicklungen wären notwendig (WAGNER *et al.* 2013). Dies sollte auch nicht Gegenstand dieser Risikobewertung sein. Vielmehr ging es darum, einen ersten, überaus wichtigen Schritt zu tätigen, nämlich eine grobe Abschätzung des Expositionsrisikos der Anhang II-Amphibienarten gegenüber Pestiziden in ihren SAC. Da dieses Expositionsrisiko artspezifisch aber auch etwa in den SAC der einzelnen Mitgliedsstaaten variieren kann, wurde eine GIS-basierte Risikobewertung durchgeführt, welche den Anteil von Landnutzungsformen, in welchen regelmäßig Pestizide appliziert werden, in den gesamten und nationalen SAC jeder Art aufzeigt. Zusammen mit einem artspezifischen Habitatexpositionsindex, auf Grundlage des tatsächlichen Vorkommens in der Kulturlandschaft sowie der Biologie und Ökologie der Art, wurde damit an folgende Schlüsselfrage herangegangen:

„Wie hoch ist das artspezifische Pestizid-Expositionsrisiko und gibt es nationale Unterschiede?“

Viele Anhang II-Amphibienarten sind laut aktueller Roten Liste der IUCN nicht in ihren Gesamtbeständen gefährdet (IUCN-Kategorie „*least concern*“: n = 11), was wohl hauptsächlich damit zusammenhängt, dass viele Arten der Anhänge der Berner Konvention einfach in die Anhänge der FFH-Richtlinie übernommen wurden, ohne ihren aktuellen Gefährdungsstatus explizit zu berücksichtigen (HOCHKIRCH *et al.* 2013). Andere wiederum sind nach der IUCN-Bewertung „*near threatened*“ (n = 6) oder sogar akut vom Aussterben bedroht („*vulnerable*“: n = 6; „*endangered*“: n = 1; „*critically endangered*“: n = 1). Zudem sind vier Amphibienarten als „prioritäre Arten“ der FFH-Richtlinie gelistet (EU 1992). Daher stellte sich die zudem Frage:

„Wie hoch ist das Pestizid-Expositionsrisiko der Arten, welche in ihren Gesamtbeständen gefährdet sind, sowie der prioritären Arten der FFH-Richtlinie?“

Die Ergebnisse dieser Studie wurden in der Fachzeitschrift „*Biological Conservation*“ der „*Society for Conservation Biology*“ (SCB) publiziert und auf dem *50. Deutschen Herpetologentag* (Bonn, 02.10.2014) vorgetragen.

Das **zweite Kapitel** der vorliegenden Arbeit beinhaltet die Ergebnisse der durchgeführten Laborexperimente mit zwei Anuren-Modellorganismen.

Laborexperimente mit den beiden Anuren-Modellorganismen *Xenopus laevis* und *Discoglossus scovazzi*

Amphibien gehören in keinem Land der Welt zu den Standardtestorganismen an denen toxikologische Experimente durchgeführt werden, welche für die Zulassung von Pflanzenschutzmitteln notwendig sind; der Schutz von wildlebenden Amphibien soll durch Ergebnisse aus Versuchen mit Stellvertreterorganismen sichergestellt werden, was oftmals bezweifelt wird (QUARANTA *et al.* 2009; BRÜHL *et al.* 2011, 2013; RELYEA 2011). In der Fachpresse wird derzeit v.a. in Frage gestellt, ob Säugetiere und Vögel ausreichende Stellvertreter für die Effekte von Pestiziden auf terrestrische Lebensstadien von Amphibien (d.h. Metamorphlinge, Juvenile und Adulti) sind (z.B. BRÜHL *et al.* 2011, 2013; RELYEA 2011) – was besonders auf die hochpermeable Haut von Amphibien zurückzuführen ist, weswegen sie Pestizide dermal viel schneller absorbieren als etwa Säuger (QUARANTA *et al.* 2009) und Pestizide den Ionentransport der Amphibienhaut stark beeinflussen und mit grundlegenden zellulären Mechanismen interagieren (BELLANTUONO *et al.* 2014). Umgekehrt scheint es eher einen Konsens darüber zu geben, dass ökotoxikologische Ergebnisse aus Versuchen mit aquatischen Invertebraten und Fischen genügen, um – in Verbindung mit Sicherheitsfaktoren – die Effekte von Pestiziden auf aquatische Lebensstadien von Amphibien (Embryonen und Larven der Amphibienarten, die zur Reproduktion an Wasser gebunden sind) abschätzen zu können (z.B. ALDRICH 2009; WELTJE *et al.* 2013). Solche Vergleiche beziehen sich jedoch häufig auf die aktiven Wirkstoffe der Pflanzenschutzmittel (ALDRICH 2009; WELTJE *et al.* 2013) und nicht auf die tatsächlich im Feld applizierten Formulierungen, welche aufgrund der ihnen zugesetzten Stoffe oftmals signifikant toxischer als der aktive Wirkstoff alleine sind (MANN & BIDWELL 1999; COX & SURGAN 2006).

Als Testsubstanz der im Rahmen dieser Doktorarbeit durchgeführten Laborexperimente wurde ein Herbizid ausgewählt, da zu Herbiziden im Vergleich zu etwa Insektiziden bisher weniger amphibientoxikologische Untersuchungen vorliegen, obwohl Herbizide besonders in der Landwirtschaft in viel größeren Mengen appliziert werden (WEIR *et al.* 2012). Mit dem ausgewählten Herbizid Focus® Ultra wurden bisher noch keinerlei amphibientoxikologische Versuche durchgeführt. Focus® Ultra besitzt den aktiven Wirkstoff Cycloxydim (CAS 101205-02-1). Es handelt sich um ein selektives Herbizid, welches im Anbau von etwa Raps oder Kartoffeln gegen Gräser angewendet wird (EFSA 2012). Cycloxydim verhindert die Acetyl-CoA Carboxylase in monokotylen Pflanzen, während es bei dikotylen Pflanzen nicht wirkt (BURTON *et al.* 1989). Seit Beginn des Jahrtausends wird es jedoch auch zunehmend beim Anbau von bestimmten Maishybriden appliziert (Mais ist eine monokotyle Pflanze und gehört der Familie der Süßgräser, Poaceae, an), welche cycloxydimresistent gezüchtet wurden. Foglich kann es bei der Kultivierung dieser Pflanzen nicht nur zu Beginn, sondern theoretisch über die gesamte Vegetationsphase gegen Ackerwildkräuter und sonstige unerwünschte Pflanzen appliziert werden (VANCETOVIC *et al.* 2009). Dies erhöht das Risiko, dass Nichtzielorganismen (wie etwa Amphibien) mit dem Herbizid in Kontakt kommen. Da Focus® Ultra – wie fast allen Pestiziden – neben dem aktiven Wirkstoff Zusatzstoffe beigemengt sind (etwa Netzmittel, welche als Vehikel dienen), wurde die Formulierung, welche auch tatsächlich im Feld appliziert wird, und nicht nur der aktive Wirkstoff getestet, da die Zusatzstoffe oftmals toxischer als der Wirkstoff alleine sind (MANN & BIDWELL 1999; COX & SURGAN 2006). Focus® Ultra enthält laut Herstellerinformation 100 g Cycloxydim pro Liter (= 10,8%) und besteht zudem – neben Wasser – aus 50% Lösungsbenzol (Solvent Naphtha) (CAS 64742-94-5) und 4% Docusat-Natrium (CAS 577-11-7).

Es wurden Laborversuche mit Embryonen und Larven zweier Anuren-Modellorganismen durchgeführt: dem Afrikanischen Krallenfrosch (*Xenopus laevis*) sowie dem Marokkanischen Scheibenzüngler (*Discoglossus scovazzi*). *X. laevis* diente bereits in vielen wissenschaftlichen Studien und Standardverfahren als Stellvertreter für andere aquatische Organismen, besonders andere Anuren, weil er bei Studien im Vergleich zu anderen getesteten Arten oft hochsensitiv gegenüber Pestizidexposition reagierte und zudem leicht im Labor gezüchtet werden kann (SCHUYTEMA *et al.* 1991; BANTLE *et al.* 1998, 1999; SCHUYTEMA & NEBEKER 1998, 1999; MANN & BIDWELL 2000; EDGINTON *et al.* 2004). Die hohe Sensitivität von *Xenopus*-Larven ist wohl hauptsächlich dadurch begründet, dass diese – wie die Larven aller Anuren aus der Familie der Pipidae – obligate Filtrierer darstellen, weshalb sie große Mengen Wasser durch ihren Buccopharynx pumpen, was wiederum einen erhöhten Kontakt gegenüber im Wasser gelösten Stoffen bedingt (VIERTEL 1990, 1992). *D. scovazzi* zählt nicht zu den Pipiden, sondern gehört der Familie der Alytidae an. Seine Larven sind nur fakultative Filtrierer, weshalb sie wohl weniger stark gegenüber im Wasser gelösten Stoffen exponiert sind (VIERTEL 1992). Diese Nicht-Pipiden-Art wurde auch daher zu Vergleichszwecken ausgewählt, da die meisten Anuren (einschließlich aller einheimischen Arten) Nicht-Pipiden darstellen.

In den ersten Versuchen wurden die akuttoxischen Effekte von Focus® Ultra auf Embryonen und Larven der beiden Arten untersucht. Die gewonnenen Ergebnisse wurden mit publizierten Ergebnissen aus Experimenten mit Standardtestorganismen verglichen, um auf folgende Fragestellung einzugehen:

„Genügen Standardtests mit aquatischen Stellvertreterorganismen in Verbindung mit Sicherheitsfaktoren, um das Risiko <u>aller</u> Pestizide auf die aquatischen Lebensstadien von Anuren abzuschätzen?“

Des Weiteren zeigte sich in bisherigen amphibientoxikologischen Versuchen, dass es signifikante Unterschiede in der Sensitivität gegenüber Testsubstanzen von Embryonen und Larven geben kann (z.B. EDGINTON *et al.* 2004; HOWE *et al.* 2004).

„Zeigen sich Unterschiede in der Sensitivität von Embryonen und Larven?“

Auch in der Sensitivität von aquatischen Lebensstadien verschiedener Anurenarten zeigten sich in manchen Studien signifikante Unterschiede (z.B. MANN & BIDWELL 1999; RELYEA & JONES 2009; WILLIAMS & SEMLITSCH 2010; FUENTES *et al.* 2011).

„Zeigen sich Unterschiede in der Sensitivität von den aquatischen Lebensstadien der beiden Anurenarten?“

Akuttoxisch wirkende (zumeist relativ hohe) Konzentrationen von Pestiziden kommen in der Natur seltener vor als niedrigere, zumeist als „subletal“ bezeichnete (STRUGER *et al.* 2008; BATTAGLIN *et al.* 2009). Folglich sind deren Effekte auf Anurenlarven naturschutzfachlich von höherer Relevanz. Für glyphosatbasierte Herbizide etwa stellen die Einflüsse subletaler Konzentrationen auf die Entwicklung von Anurenlarven (wie etwa Zeit bis und Größe zur Metamorphose: HOWE *et al.* 2004; WILLIAMS & SEMLITSCH 2010) den wohl sensibelsten Endpunkt dar (WAGNER *et al.* 2013). Daher stellte sich zu dem bisher in amphibientoxikologischen Studien nicht getesteten cycloxydimbasierten Herbizid Focus® Ultra analog die Frage:

„*Welche Effekte lösen subletale Konzentrationen des Herbizids Focus® Ultra auf die Entwicklung von* X. laevis *aus?*“

Zudem legen amphibientoxikologische Studien nahe, dass unterschiedliche Larvalstadien – zumeist klassifiziert nach dem System von GOSNER (1960), im Falle von *X. laevis* nach dem speziellen System von NIEUWKOOP & FABER (1956) – unterschiedlich stark auf

Pestizidexposition reagieren (BRIDGES 2000; GREULICH & PFLUGMACHER 2003; BOONE *et al.* 2013; BIGA & BLAUSTEIN 2013). Daher wurde in einem Langzeitversuch die gesamte Larvalentwicklung von *X. laevis* betrachtet, Larven jedoch nicht nur regelmäßig, sondern auch explizit zu ausgewählten Entwicklungsstadien gegenüber einer subletalen Dosis des Herbizids exponiert.

„*Wie reagieren unterschiedliche Larvalstadien von* X. laevis *auf subletale Konzentrationen des Herbizids Focus® Ultra?*"

Es wurde ein frühes und ein mittleres Larvalstadium (NIEUWKOOP & FABER-Stadium [NF] 47 und 53) ausgewählt, um eine steile und eine weniger steile Wachstumsphase zu prüfen und dazu ein Prometamorphosestadium (NF 57) mit geringem Längenwachstum aber Entwicklung und Wachstum der Extremitäten (Abb. 2). Im Falle des getesteten Herbizids Focus® Ultra sind diese unterschiedlichen Expositionsszenarien auch daher interessant, da es beim Anbau cyloxydimresistenter Maishybride mehrfach appliziert werden kann (VANCETOVIC *et al.* 2009) und folglich unterschiedliche Entwicklungsstadien von Anuren potenziell mit ihm in Kontakt kommen können.

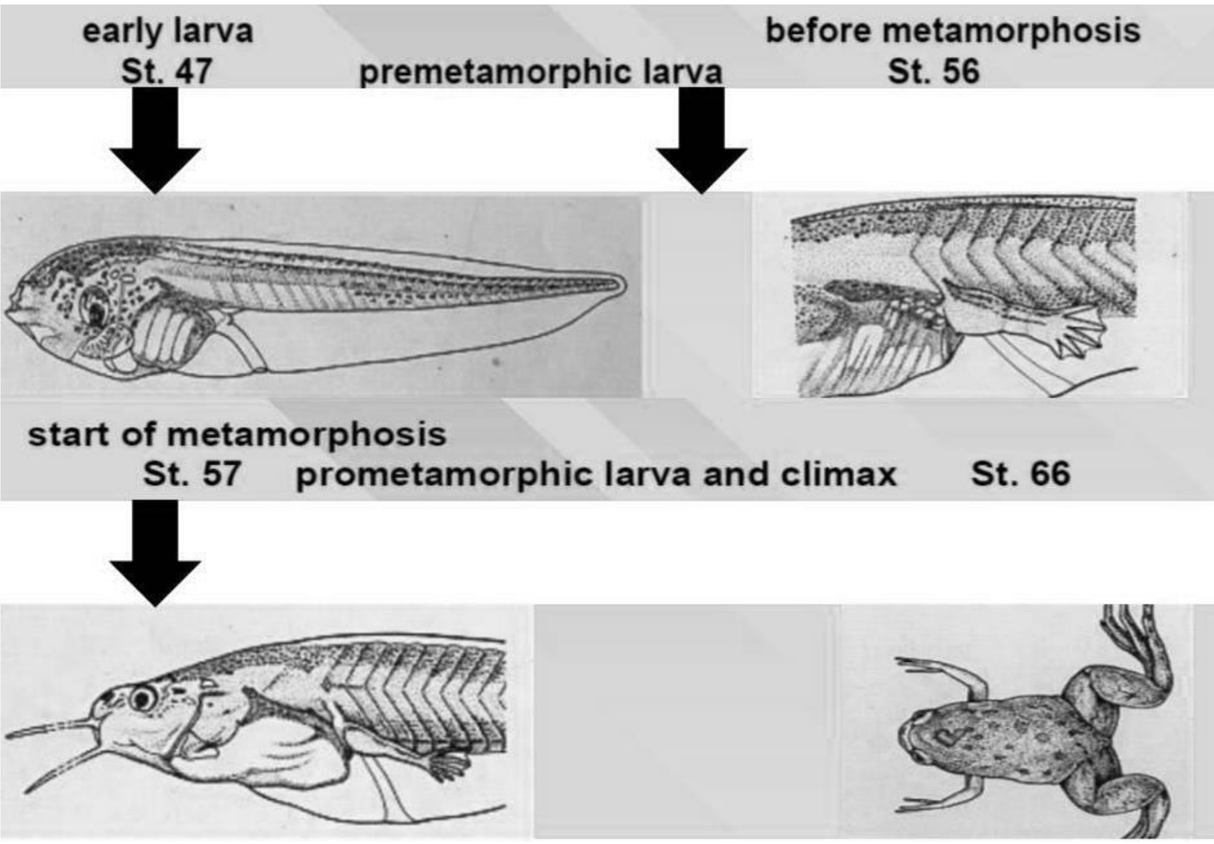

Abb. 2: Expositionszeitpunkte (Pfeile) unterschiedlicher Entwicklungsstadien von *X. laevis* gegenüber dem Herbizid Focus® Ultra im durchgeführten Langzeitversuch (nach B. VIERTEL, unpubl.).

Das erste unpublizierte Manuskript zu den durchgeführten Laborversuchen behandelt die akuttoxischen Effekte von Focus® Ultra auf Embryonen und frühe Larven von *X. laevis* und befindet sich derzeit noch im „Peer-Review-Prozess“ bei der Fachzeitschrift „*Bulletin of Environmental Contamination and Toxicology*“. Das zweite unpublizierte Manuskript beinhaltet die akuttoxischen Effekte von Focus® Ultra auf Embryonen und frühe Larven von *D. scovazzi* und ist im „Peer-Review-Prozess“ bei der Zeitschrift „*Archives of Environmental Contamination and Toxicology*“. Das dritte unpublizierte Manuskript beinhaltet die Ergebnisse des Langzeitversuches mit *X. laevis.*

Das **dritte Kapitel** der vorliegenden Arbeit beschäftigt sich mit den beobachteten Effekten von Pestiziden auf Amphibien im Freiland.

Rezente Einflüsse landwirtschaftlicher Nutzung auf die Ausbildung von Deformationen bei frühen Entwicklungsstadien des Grasfrosches (*Rana temporaria*)

Wenn Pestizide in Gewässer gelangen, wirken in der Natur – im Gegensatz zu den standardisierten Bedingungen im Labor – viele weitere Faktoren, welche Effekte dieser sowohl direkt als auch indirekt beeinflussen können (RELYEA 2009; JONES *et al.* 2010, 2011). Bstimmte Sedimente können sich etwa positiv auswirken, indem Pestizide schnell an diese adsorbieren und so weniger schädlich wirken können (BERNAL *et al.* 2009), während dies bei schnell toxisch wirkenden Substanzen zumeist nicht ausreichend ist (RELYEA 2011). Prädatoren können durch Anurenlarven wahrgenommen werden und dieser zusätzliche Stress kann die letalen Effekte von Pestiziden stark erhöhen (RELYEA & MILLS 2001; RELYEA 2005; BRODMAN *et al.* 2010). Dies gilt auch für Pestizidmixturen, welche im Freiland wahrscheinlich sind, da selten ein einziges Pestizid in der modernen Landwirtschaft Anwendung findet (BRODEUR *et al.* 2014). Auch können Unterschiede im pH-Wert des Wassers die Effekte von Pestiziden auf Anurenlarven formulierungsabhängig stark beeinflussen (EDGINTON *et al.* 2004). Bei höheren pH-Werten liegen etwa tallowaminhaltige Netzmittel größtenteils in ihrer nicht-ionisierten Form vor, welche die Bioakkumulation in den Kiemen der Larven beschleunigt (EDGINTON *et al.* 2004). Umgekehrt begünstigt saures Milieu die Aufspaltung anderer Pestizide in Bestandteile, welche besser von Larven aufgenommen werden können, weshalb hier niedriger pH negativ wirkt (EDGINTON *et al.*

2003). Des Weiteren werden durch narkotisierte oder deformierte Tiere, deren Fluchtverhalten beeinträchtigt ist, leichte Beute von Prädatoren (BERNABÒ *et al.* 2011; WAGNER *et al.* 2014). Diese Aufzählung lässt sich fortführen. Daher sind Feldstudien zu den Effekten des Einsatzes von Agrochemikalien auf Amphibien von hohem naturschutzfachlichem Wert.

Ein neues Methodenhandbuch des VDI (VDI 2013) beschäftigt sich mit dem Monitoring der Effekte des Anbaus gentechnisch veränderter Organismen auf die benachbarte Flora und Fauna; in diesem findet sich auch eine Richtlinie zum Amphibien-Monitoring, welche besonders darauf abzielt, potenzielle Auswirkungen durch die Veränderungen im Spritzregime aufzuzeigen (VDI 4333; siehe BÖLL *et al.* 2013). Bis zum Beginn meiner Promotionszeit waren PD Dr. STEFAN LÖTTERS, Prof. Dr. MICHAEL VEITH und ich Mitglieder des VDI-Ausschusses zur Erstellung der besagten Richtlinie, weshalb das BfN an uns herantrat, um das in dieser Richtlinie beschriebene Larvenmonitoring erstmals im Freiland zu testen, damit eventuelle Änderungen noch in den Druck der Richtlinie einfließen können. Dieses Larvenmonitoring zielt hauptsächlich darauf ab, Deformationsraten bei Anurenlarven zu erfassen. Die Ausbildung von Missbildungen durch Umweltchemikalien ist im Labor ein relevanter Endpunkt, um die teratogenen Effekte zu studieren („Teratogenic Concentration“; TC). Eine Deformationsrate über 5% sollte im Freiland – auf Grundlage der vorhandenen Literatur zu diesem Themenfeld – als unnatürlich und anthropogen bedingt erachtet werden (COOKE 1981; PIHA *et al.* 2006). Im Rahmen dieser Freilandarbeit waren frühe Entwicklungsstadien (Larven aber auch Metamorphlinge) des Grasfrosches (*Rana temporaria*) Untersuchungsgegenstand und es konnten – neben dem reinen Praxistest der Monitoringvorgaben – mehrere wissenschaftliche Fragestellungen bearbeitet werden. Zum einen wurden mehr Parameter aufgenommen als in der Richtlinie vorgeschrieben sind, um mit einer statistischen Auswertung deren Erklärungskraft bezüglich der Wahrscheinlichkeit der Ausbildung von Deformationen zu testen. Es stellte sich zudem die grundsätzliche Frage, ob tatsächlich eine Korrelation zwischen der die Laichgewässer umgebenden Landnutzung (Landnutzung mit regelmäßigem Einsatz von Agrochemikalien oder aber natürliche Gewässer) und der Deformationsraten bei Anurenlarven besteht.

„Sind Anurenlarven aufgrund der Ausbildung von Deformationen als Bioindikatoren für die Belastung von Kleingewässern mit Agrochemikalien geeignet?“

„Bedingt die die Laichgewässer umgebende Landnutzung die Wahrscheinlichkeit der Ausbildung von Deformationen bei Anurenlarven?“

Des Weiteren wurde überprüft, ob der in der Literatur beschriebene „5% Schwellenwert“ für die anthropogen bedingte Ausbildung von Deformationen (COOKE 1981; PIHA *et al.* 2006) tatsächlich Allgemeingültigkeit besitzt.

„Wird der 5%-Schwellenwert in den Untersuchungsgebieten nur in den anthropogen beeinflussten Gewässern überschritten?“

Da mehrere Studien zeigten, dass sich Agrochemikalien auf das Wachstum und die Entwicklung von Amphibien auswirken (CAUBLE & WAGNER 2005; RELYEA & DIECKS 2008; BRODEUR *et al.* 2011; ATTADEMO *et al.* 2014), wurden zudem späte Larvalstadien (kurz vor der Metamorphose) standardisiert in ihrem Ursprungswasser bis zur Metamorphose zwischengehältert, um auf folgende Frage einzugehen:

„Beeinflusst die die Laichgewässer umgebende Landnutzung Entwicklung und Wachstum bis zur Metamorphose?“

Diese Ergebnisse wurden in der Fachzeitschrift „*Ecological Indicators*“ publiziert und auf zwei Tagungen präsentiert (*49. Deutscher Herpetologentag*, Bonn, 26.09.2013; *Amphibian Conservation Research Symposium*, London, 11.05.2014).

Verhaltensversuche zur potenziellen Meidung von kontaminierten Gewässern durch Amphibien

Manche Amphibien scheinen mit Pestizidrückständen kontaminierte Gewässer zu meiden (TAKAHASHI 2007; VONESH & BUCK 2007; VONESH & KRAUS 2009). Durch umweltrelevante Kontamination können so Fortpflanzungsstätten zerstört oder beschädigt werden. Zu diesem Thema wurden bisher nur sehr wenige Studien durchgeführt (TAKAHASHI 2007; VONESH & BUCK 2007; VONESH & KRAUS 2009) und keine einzige befasste sich mit in Europa vorkommenden Arten noch mit für Europa relevanten Umweltkonzentrationen. Alle durchgeführten Studien wiesen eine Meidung kontaminierter Gewässer nach, jedoch teils nur von bestimmten Arten (VONESH & KRAUS 2009). Effekte wurden den Netzmitteln in den Herbizidformulierungen zugewiesen, weshalb neben einer glyphosatbasierten Formulierung (Roundup LB PLUS®) der aktive Wirkstoff Glyphosat und dessen Hauptmetabolit AMPA in Verhaltensversuchen mit drei einheimischen Amphibienarten (Grasfrosch [*Rana temporaria*], Fadenmolch [*Lissotriton helveticus*] und Bergmolch [*Ichthyosauara alpestris*]) im Freiland eingesetzt wurden. Da sich Arenen in Verhaltensversuchen mit Amphibien bewährt haben (z.B. LANDLER & GOLLMANN 2011), wurden solche erstmalig im Hinblick auf diese spezifische Fragestellung eingesetzt und dafür auf dem Testgelände der Geobotanik (Universität Trier) aufgebaut.

Die grundlegenden Fragestellungen der durchgeführten Verhaltensversuche lauteten:

„Meiden einheimische Amphibien Gewässer, welche mit umweltrelevanten Konzentrationen eines glyphosatbasierten Herbizids, seines aktiven Wirkstoffes oder dessen Hauptmetaboliten kontaminiert wurden?“

„Kommt es zu substanz- und artspezifischen Reaktionen?“

Diese Ergebnisse wurden in der Fachzeitschrift *„Archives of Environmental Contamination and Toxicology“* publiziert und auf drei Tagungen präsentiert (*SEH European Congress of Herpetology*, Veszprém (Ungarn), 22-27.08.2013; *International Conference on Environmental Indicators*, Trier, 17.09.2013; *49. Deutscher Herpetologentag*, Bonn, 26.09.2013).

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Kapitel I: Risikobewertungen

Questions concerning the potential impact of glyphosate-based herbicides on amphibians

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Abstract

Use of glyphosate-based herbicides is increasing worldwide. The authors review the available data related to potential impacts of these herbicides on amphibians and conduct a qualitative meta-analysis. Because only a little is known about environmental concentrations of glyphosate in amphibian habitats, and virtually nothing about environmental concentrations of the substances added to the herbicide formulations that mainly contribute to adverse effects, glyphosate levels can only be seen as approximations for contamination with glyphosate-based herbicides. The impact on amphibians depends on the herbicide formulation with different sensitivity of taxa and life-stages. Effects on development of larvae apparently are the most sensitive endpoints to study. As with other contaminants, costressors mainly increase adverse effects. If and how glyphosate-based herbicides and other pesticides contribute to amphibian decline is not answerable yet due to missing data on how natural populations are affected. Amphibian risk assessment can only be conducted case-specifically, with consideration of the particular herbicide formulation. The authors recommend better monitoring of both amphibian populations and contamination of habitats with glyphosate-based herbicides, not just glyphosate, and suggest including amphibians in standardized test batteries to study at least dermal administration.

Keywords: amphibian decline, aminomethylphosphonic acid, polyethoxylated alkylamine, Roundup, pesticide

Introduction

Glyphosate (GLY) is the active ingredient in many nonselective (or broad-spectrum) herbicide formulations. It has mortal effects on most species of green plants by inhibiting the monomeric enzyme 5-enolpyruvylshikimate-3-phosphate synthase, the key enzyme of the shikimate pathway for the biosynthesis of aromatic amino acids (GIESY *et al.* 2000; DILL 2005; DILL *et al.* 2010). Also, GLY is known to be a strong systemic metal chelator (TOY & UHING 1964), which can impair micronutrient uptake in plants and reduce their growth (CAKMAK *et al.* 2009; JOHAL & HUBER 2009). GLY-based herbicides (GBH) - for instance, different formulations under the brand name Roundup® - are applied in no-tillage farming in conventional agriculture, winegrowing, forest management, noncultivated areas and other areas; likewise, GBH are applied in private gardening (DILL 2005; DILL *et al.* 2010). However, the main use of GBH is in the cultivation of genetically modified crops that are made herbicide-resistant (HR). That is, they resist nonselective herbicides at a degree lethal to weeds. Due to the growing importance of HR crops, GBH are dominating the worldwide herbicide market (DUKE & POWLES 2008). Taking the United States as an example, the GLY sales volume has significantly increased since 1996, the year when HR crop cultivation began, from about 25 million pounds to 180 million pounds of active ingredient in 2007 (ASPELIN 1997; GRUBE *et al.* 2011). Against the background that the worldwide use of GBH is increasing and unparalleled in modern weed management, a comparison of ecotoxicological endpoints (like median lethal concentration [LC50] values) of GLY and GBH with those of other pesticides and contaminants (BRAIN & SOLOMON 2009) is a necessary step; however, it is by far insufficient to assess and compare the impacts of different herbicides to amphibians (RELYEA 2011).

HR systems consist of an HR crop and its associated complementary herbicide, which is usually nonselective (GBH or others). HR systems are propagated to result in a net reduction of herbicide use compared with conventional cropping systems. While a large 2-yr field experiment supports this view (CHAMPION *et al.* 2003), agricultural practice in the first years since adoption in the United States rather indicate no significant decrease in amounts of active ingredients (HEIMLICH *et al.* 2000). A study that covered the whole adoption period of genetically modified crops in the United States concluded that HR systems actually increased herbicide use between 1996 and 2011 (BENBROOK 2012). According to the study, which was based on National Agricultural Statistics Service survey data, herbicide use increased by 7%. Herbicide increase seems to be the case in unsustainable monocultures, where rotation of crops and traits is disregarded and increasing resistance of weeds creates a demand for higher amounts of nonselective herbicides as well as the reuse of selective herbicides (BENBROOK 2012). The number of HR weeds has increased since the 1980s (see http://www.weedscience.org/chronIncrease.gif). In case of GLY, a total of 23 GLY-resistant weeds have been identified worldwide as of July 2012 (http://www.weedscience.org/summary/MOASummary.asp).

The environmental safety of GLY, compared to the herbicides it replaces, is frequently highlighted (HEIMLICH *et al.* 2000). Based on official ecotoxicological standard tests, GLY is considered to be 'practically non-toxic' to 'slightly toxic' to animals (USEPA 1993; EC 2002; DILL *et al.* 2010). However, this information cannot be simply 'transferred' to GBH because some added substances occasionally are more toxic than the active ingredient itself, GLY (COX & SURGAN 2006; DILL *et al.* 2010). Higher toxicity of GBH compared to GLY, especially to organisms in the aquatic environment, is mainly caused by surfactants that are added for better entry of the active ingredient into the plant tissue (DILL *et al.* 2010).

Amphibians are not standard test organisms in official studies, and usually results from fish and aquatic invertebrates (for aquatic amphibian life stages), birds, and mammals (for terrestrial amphibian life stages) are taken as proxies (RELYEA 2011). Both the absence of amphibians from such tests and the transferring of results from other animal groups to amphibians are questionable for 3 reasons. First, worldwide, amphibians are dramatically declining with more than one-third of all known (~7000) species threatened with extinction. Within the last 3 decades, we have witnessed species and population declines and extinctions at alarming rates, and environmental contaminants are supposed to play a role (MENDELSON *et al.* 2006; STUART *et al.* 2008). Second, most amphibian species are ecologically unrivaled among vertebrates by spending part of their early life in water, while later (adult) stages are on land. Consequently, these organisms face an unparalleled risk of contamination with GBH (and other pesticides) in both the aquatic and the terrestrial milieus. Several ways of exposure are possible such as overspraying (RELYEA 2005a; BERNAL *et al.* 2009a), runoff after downpour (EDWARDS *et al.* 1980; PERUZZO *et al.* 2008), airborne drift (DAVIDSON *et al.* 2001; DAVIDSON *et al.* 2002; DAVIDSON 2004), and contact with residues on soil and plant material (BRÜHL *et al.* 2011). Moreover, amphibians may incorporate contaminants via their larval plant and adult insect diets (MCCOMB *et al.* 2008). Finally, amphibians are biologically unique among all vertebrates. They are the only tetrapods with an embryo not protected by an amnion cavity and with a free-swimming larva. In addition, they are the only Anamnia (*i.e.*, fishes and amphibians) which breathe through well-developed lungs and have a highly permeable skin used for relevant uptake from the environment (DUELLMAN & TRUEB 1986). For these reasons, it may be grossly misleading when conclusions gained in tests with warm-blooded tetrapods (mammals and birds) are transferred to amphibians. Test substances are administered orally to mammals and birds (RELYEA 2011); but for amphibians dermal uptake seems more important. Xenobiotics can diffuse into amphibians 1 or 2 orders of magnitude faster (depending on the species' hydrophobicity) than into mammals (QUARANTA *et al.* 2009). Although most tests with teleost fish were conducted with larval forms that also have a permeable skin, transferring those results to amphibian larvae may also be misleading because amphibian species differ considerably in their response to herbicides. The present study deals, among other things, with the question of which cases data from standard test organisms may be transferred to amphibians.

When it comes to GBH concentrations in the environment that are relevant to amphibians, experimental studies draw inconsistent conclusions. Numerous studies are in agreement that at least some GBH – in particular those with the surfactant polyethoxylated tallowamine or polyethoxylated alkylamine (POEA) – can have substantial lethal or sublethal effects on amphibians in the environment. Other studies have found deleterious effects on amphibians as well, but concluded no risk from the use of POEA-containing GBH under realistic environmental exposures and 'normal-use' scenarios. Results mainly refer to frogs and toads (Anura) since the other 2 amphibian groups (Caudata, Gymnophiona) remain underrepresented in studies. Up to now, amphibian experiments have shown considerable variation in terms of their aims, applied designs (GBH formulation, concentration, renewal of test media, etc.), and the species and life stages examined, which makes it a challenge to directly compare them. The purpose of the present study is to review the available literature and conduct a qualitative meta-analysis concerning the potential effects of GLY and GBH on specific endpoints. We consider such a synthesis important against the background of the increasing use of GBH, the different biology of amphibians and test organisms, and the ongoing amphibian decline. Furthermore, we understand amphibians as wild animals that persist in agricultural landscapes because, in many countries, primary amphibian habitats such as natural wetlands and floodplains have been mainly destroyed to expand the arable land (MANN *et al.* 2009).

Environmental fate and concentrations of GBH, GLY and AMPA

First at all, it is most important to realize that we know next to nothing about the environmental concentrations of surfactants and adjuvants used in GBH. Therefore, GLY concentrations can only be used as approximations for contamination with GBH.

In both soil and water, GLY is almost exclusively degraded by microorganisms to its main metabolite aminomethylphosphonic acid (AMPA) and eventually to carbon dioxide (GIESY *et al.* 2000). GLY and AMPA are rapidly immobilized by adsorption to soil and sediment. Because this is highly dependent on soil type, temperature, etc., half-life values of GLY in soil can range from a few to over 200 d (FENG *et al.* 1990; GIESY *et al.* 2000; MORILLO *et al.* 2002; BORGGAARD & GIMSING 2008). AMPA shows a significantly stronger adsorption behavior (MAMY *et al.* 2010), making it less available to microorganisms (RUEPPEL *et al.* 1977) and leading to commonly longer half-life values (~80-900 d GIESY *et al.* 2000) than for GLY. In water, half-life values of both GLY and AMPA are estimated to range from 7 d to 14 d (GIESY *et al.* 2000). GLY competes with inorganic phosphate for soil binding sites (DION *et al.* 2001; GIMSING & BORGGAARD 2002a; GIMSING & BORGGAARD 2002b), and adsorbed GLY can be remobilized by phosphate fertilization (BOTT *et al.* 2011). For AMPA, long-term leaching from fields that were treated with GBH years before has been reported (KJAER *et al.* 2005). In general, it should be understood that although GLY is the main source of AMPA, it is not the only source because other phosphonate compounds are degraded to AMPA as well (SKARK *et al.* 1998). Since many amphibian species choose nonflowing, small, and shallow water bodies for reproduction, data on GLY and AMPA concentrations in these kinds of water bodies are more relevant than in large, deep, or flowing waters (RELYEA 2006; MANN *et al.* 2009). The rate of GLY dissipation from such small water bodies is mainly a function of the local conditions and should be considered site-specifically (GIESY *et al.* 2000).

Although data about the concentration of GLY and AMPA in soil and sediments are available, risks to amphibians cannot be well assessed because specific studies are missing. Negative effects on amphibians by simple contact with contaminated soil have been suggested for pesticides other than GLY (BAKER 1985). Although both GLY and AMPA should be strongly adsorbed to the soil for most of their time (compared with other substances), it should not be dismissed that terrestrial life stages of amphibians may suffer from contact with soil (or plant material) contaminated with GLY (BRÜHL *et al.* 2011). Furthermore, many amphibian larvae incorporate sediment during feeding (DUELLMAN & TRUEB 1986) (*e.g.*, by grazing algae);

how much soil they take up and whether adsorbed pesticides become remobilized in the larvae has not been investigated so far.

Herbicides can be wind-drifted from the field into aquatic and terrestrial habitats during application (TSUI & CHU 2008), but the scale is largely unknown (DAVIDSON *et al.* 2001; DAVIDSON *et al.* 2002; DAVIDSON 2004). Maximum drift rates of 9.1 ng/m³ for GLY and 0.97 ng/m³ for AMPA were reported (CHANG *et al.* 2011). It is notable that both GLY and AMPA were also found in rainfall (SCRIBNER *et al.* 2007; CHANG *et al.* 2011) and even in mini-pools of rainforest plants (phytotelmata) (KAISER 2011). The concentrations in rain, reported for the first time, were generally low (GLY maximum of 2.5 µg acid equivalent [a.e.]/L; AMPA maximum of 0.48 µg/L (CHANG *et al.* 2011) but demonstrate that environmental contamination of GLY by drift and exposure to amphibians in this way is a realistic scenario (KAISER 2011).

Concentrations in water: It is notable that in water, AMPA was detected more frequently than GLY but usually at much lower concentrations (SCRIBNER *et al.* 2007; PERUZZO *et al.* 2008; STRUGER *et al.* 2008). This is because AMPA stays longer in soil and sediment than GLY and gradually leaches from it into the water body (KJAER *et al.* 2005). Only 1 study reported similar concentrations for both GLY and AMPA (maximum of ~0.4 mg/L (COUPE *et al.* 2012) in a subsurface drain, which is not an amphibian habitat. Due to its relatively rapid dissipation, it can be assumed that most GLY values measured directly in the environment are probably below the peak concentrations present directly after herbicide applications or after downpour. However, knowing maximum concentrations – even if they prevail for short periods only – is crucial because most studies observed acute toxic effects of GBH on tadpoles, that is, immediately within the first 24 h after tadpoles were exposed to the GBH (SMITH 2001; TRUMBO 2005; COMSTOCK *et al.* 2007; RELYEA 2011).

Beacuse analysis of GLY is more expensive and challenging than for many other pesticides – which can be determined simultaneously by gas chromatography-mass spectrometry (WILLIAMS & SEMLITSCH 2010) – the data on environmental concentrations of GLY are generally sparse, especially for small, shallow water bodies that are often inhabited by amphibians (STRUGER *et al.* 2008; BATTAGLIN *et al.* 2009). Water bodies are directly oversprayed when aquatic weeds are combated (BATTAGLIN *et al.* 2009), and only certain formulations, which are less-toxic, are labeled for use against aquatic weeds such as Accord®. In some countries, aerial application of GBH is practiced in agricultural HR systems and in forest management to kill broadleaf trees and favor the more marketable conifer trees (*e.g.*, in Canada the formulation Vision®, which is equivalent to Roundup Original®) (THOMPSON *et al.* 2004). It can be postulated that aerial application in agriculture and forestry is responsible for the highest GLY concentrations in aquatic habitats.

Most available data on surface water concentrations are from pesticide-monitoring programs in the United States and relate to streams or large nonflowing waters like lakes (SCRIBNER *et al.* 2007), which are not commonly used by amphibians (BATTAGLIN *et al.* 2009). For GLY, concentrations in surface waters can be divided into 3 groups according to the applied method: 1) those directly measured in the environment without intervention (available for the Americas and Europe); 2) those measured in field experiments shortly after application or in runoff during the first heavy rains (available for the Americas and Europe); 3) those estimated in worst-case scenarios (available for North America and Germany). The 5 maximum GLY concentrations range from 0.17 mg a.e./L to 0.70 mg a.e./L for group 1, 0.27 mg a.e./L to 3.10 mg a.e./L for group 2, and 1.43 mg a.e./L to 7.60 mg a.e./L for group 3 (see Table 1).

In group 1, some high concentrations derive from direct overspraying with GBH approved for aquatic use (*e.g.*, Rodeo®), but the highest concentration of 0.70 mg a.e./L is from waters near a HR soybean cultivation area (PERUZZO *et al.* 2008). In group 3, the highest expected environmental concentration (EEC) in surface water of 7.6 mg a.e./L relates to the scenario of a direct application of Roundup® (360 g active ingredient [a.i.]/L) at the maximum rate to a lentic water body of 5 cm in depth, like a flooded field (MANN & BIDWELL 1999). The worst-case EEC for GLY calculated by national agencies are usually lower (*e.g.*, 1.4 mg a.e./L for Canada: EDGINTON *et al.* [2004] and 0.9 mg a.e./L for Germany: BVL [2010a]) than those calculated by some researchers (see group 3 in Table 1).

In the EU the Water Framework Directive (Directive 2000/60/EC) provides a procedure to set Environmental Quality Standards. These are 100 µg a.e./L for GLY and 450 µg/L for AMPA (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri¼CELEX: 32000L0060:EN:HTML). Taking Germany as an example, most GBH require one to keep a distance of 5 m to 20 m from adjacent water bodies during application. A 5-m buffer strip would reduce the EEC of 0.9 mg a.e./L to approximately 0.005 mg a.e./L (BVL 2010a) (but note that only pesticide drift is calculated, not runoff or leaching). There are also some formulations such as Roundup UltraMax® and Roundup Turbo® where no distance has to be regarded, although these 2 formulations have tallowamine surfactant systems (http://www.roundup.de); but regular buffer strips between 5 m and 50 m are required in most of the different German states (http://www.umweltdaten.de/wasser/laender.pdf). This means that in most water bodies adjacent to fields the concentration of GLY should not legally exceed 5 µg a.e./L, if herbicides are applied according to the provisions. However, small, ephemeral ones like puddles, which are also used by some amphibian species for reproduction, are usually not regarded as water bodies and, therefore, are not covered by application provisions (MANN *et al.* 2003; BATTAGLIN *et al.* 2009).

Tab. 1: Top 5 maximum GLY concentrations in surface waters as reported in various studies and measured in 1) environmental samples (degradation state unknown); 2) field studies (shortly after application); and 3) estimated worst-case scenarios (expected environmental concentration or predicted environmental concentration).

Amounts given in milligram acid equivalent per liter (mg a.e./L).

Environmental sample	Field shortly after application	Worst-case expected environmental concentration	References
0.70	3.10	7.60	MANN & BIDWELL 1999; TRUMBO 2005; PERUZZO *et al.* 2008
0.43	1.95	2.90	PERKINS *et al.* 2000; THOMPSON *et al.* 2004; SCRIBNER *et al.* 2007; COUPE *et al.* 2012
0.33	1.70	2.80	GIESY *et al.* 2000; BATTAGLIN *et al.* 2009
0.29	1.24	2.70	NEWTON *et al.* 1984; SOLOMON & THOMPSON 2003; COUPE *et al.* 2012
0.17	0.27	1.43	NEWTON *et al.* 1994; WOJTASZEK *et al.* 2004; TREGOUET 2006

Methods

We searched the Web of Knowledge and Google Scholar for published scientific articles with the following key words (latest by 15 March 2012): glyphosate*, glyphosate* + amphibia*, Roundup*, Roundup* + amphibia*. Furthermore, we employed the database ECOTOX (US Environmental Protection Agency [USEPA]). We examined the references of the retrieved publications for further information. In addition, a limited number of miscellaneous gray literature, largely such distributed via national authorities, was used when relevant. The debate as to whether amphibians could be affected in their natural environment is sometimes perhaps steered by motivation other than scientific research because some authors question each other's vested interests (RELYEA 2006, 2011; THOMPSON *et al.* 2006). Nevertheless, or precisely because of this, we included all and did not exclude information based on its source. To conduct a qualitative meta-analysis, we used the vote-counting method (GUREVITCH & HEDGES 1993; ROHR & MCCOY 2010) and quantified which number of studies found or did not find significant effects ($\alpha = 0.05$) of GLY and GBH on following response variables: unnatural deformity rates of larvae, endocrine disruption in larvae, effects on development, inhibition of specific enzymes, genotoxic effects, effects on embryos, behavioral effects, abiotic interactions, and biotic interactions. We used Fisher's exact test to test against the null hypothesis that GLY and GBH do not have effects on the considered response variable. All studies on acute toxicity found dose-response relationships, and we solely descriptively show published LC50 values to compare them with environmentally relevant concentrations. Analyses were performed with the software *R* (R DEVELOPMENTAL CORE TEAM, Vienna).

Results

In total, 51 toxicological studies concerning the impact of GLY or GBH on amphibians were found; important details are summarized in Appendix A. Most studies (n = 36) investigated effects on tadpoles (*i.e.*, anuran larvae), little on embryos (n = 5), terrestrial life stages of anurans (n = 8), newts and salamanders (all life stages; n = 6), and overall, no studies at all are known on amphibians of the order Gymnophiona (caecilians). Only in an (anecdotal) field observation, terrestrial life stages of a caecilian species suffered burnlike wounds 5 h to7 h after Roundup® applications in a tea plantation in Sri Lanka (DE SILVA 2009).

Acute toxicity

Toxicological studies focused mainly on larval anurans (tadpoles) from North America. It is challenging to directly compare the various experiments because of differences in design, length, and exposure conditions. Nevertheless, some general conclusions can be drawn.

Terrestrial life stages: Generally, absorption of pesticides is faster in amphibians than other vertebrates (QUARANTA *et al.* 2009). However, effects of GLY and GBH on terrestrial life stages of amphibians have rarely been investigated. Direct overspraying at recommended application rates resulted in 79% of the tested juvenile frogs and toads from the United States dying within 24 h in 1 experiment (RELYEA 2005a) and up to 30% in another carried out on Colombian anurans (BERNAL *et al.* 2009a). Not all GBH seem to pose immediate risks for amphibians under field conditions because mortality can vary between 0% and approximately 80% depending on the formulation (DINEHART *et al.* 2009). The presence of soil or litter remarkably reduced adverse effects on oversprayed terrestrial life stages in 2 cases (BERNAL *et al.* 2009a; DINEHART *et al.* 2009). When compared with direct overspraying, exposure of adults and metamorphs of an Australian frog species to dilute GBH and GLY solutions revealed slightly different 48-h LC50 values (~50 mg a.e./L and 80 mg a.e./L, respectively) (MANN & BIDWELL 1999).

Aquatic larvae: Studies have shown that both the salt and acid form of GLY, which are used as active ingredients, are 'slightly toxic' (10 mg/L<LC50<100 mg/L) or even 'practically nontoxic' (LC50>100 mg/L) to tadpoles (BIDWELL & GORRIE 1995; MANN & BIDWELL 1999). One study stated a 96-h LC50 value as low as 6.5 mg a.e./L for GLY, but this value should be treated with caution, because it is based on tests with a GBH formulation and a surfactant, rather than with GLY directly (TRUMBO 2005). Some GBH are classed as 'slightly toxic' to 'practically nontoxic' (*e.g.*, Roundup Biactive®) (BIDWELL & GORRIE 1995; MANN & BIDWELL 1999). Conversely, other GBH (*e.g.*, Roundup Original®) have been found to be 'moderately toxic' (1 mg/L<LC50<10 mg/L) or even 'highly toxic' (0.1 mg/L<LC50<1 mg/L) to tadpoles (RELYEA & JONES 2009; KING & WAGNER 2010). Categories are those defined by the USEPA. When LC50 values of different GBH for 37 amphibian larvae species were plotted (Fig. 1), the median was about 2 mg a.e./L regardless whether the less toxic GBH, which are also mainly approved for aquatic weed management, were considered (Fig. 1A) or just moderately toxic GBH (Fig. 1B).

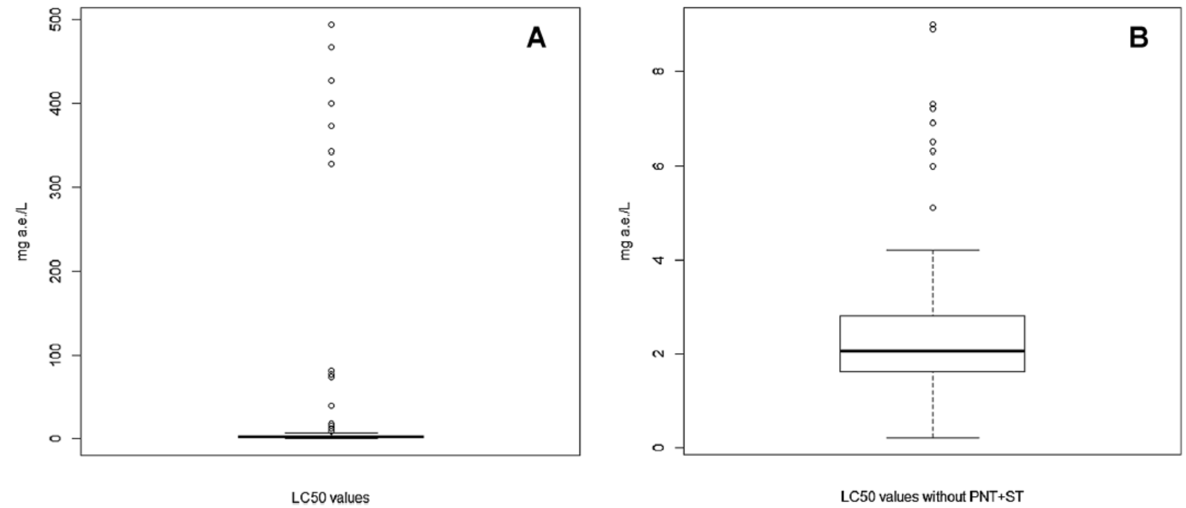

Fig. 1: Median lethal concentration (LC50) values of GBH for amphibian larvae of 37 species.

All values are in milligrams acid equivalent per liter (mg a.e./L) (MANN & BIDWELL 1999; LAJMANOVICH *et al.* 2003; EDGINTON *et al.* 2004; HOWE *et al.* 2004; RELYEA 2005b; TRUMBO 2005; BERNAL *et al.* 2009a, b; RELYEA & JONES 2009; DINEHART *et al.* 2010; KING & WAGNER 2010; FUENTES *et al.* 2011; JONES & RELYEA 2011; LAJMANOVICH *et al.* 2011).

(A) With practically non-toxic (PNT) and slightly toxic (ST) GBH (tested GBH = 18, minimum = 0.20, 1st quartile = 1.70, median = 2.15, mean = 21.70, 3rd quartile = 3.45, maximum = 494.00).

(B) Without PNT+ST GBH (tested GBH = 12, minimum = 0.20, 1st quartile = 1.60, median = 2.05, mean = 2.53, 3rd quartile = 2.80, maximum = 9.00).

The relatively wide range of toxicity seems to be associated with the different surfactants present in the GBH. Surfactants are mainly from the alkoxylated alkyl amine family (WILLIAMS & SEMLITSCH 2010). In concert with several other studies on aquatic organisms (TSUI & CHU 2003; BRAUSCH & SMITH 2007; BRAUSCH *et al.* 2007), GBH with tallowamine surfactants, especially POEA, appear to be amongst the most dangerous formulations to tadpoles (MANN & BIDWELL 1999; HOWE *et al.* 2004; RELYEA 2005a; WILLIAMS & SEMLITSCH 2010). POEA appears to contribute most to the acute toxicity, since it has been shown that POEA is more toxic than the GBH itself (and the GBH is more toxic than only GLY) (PERKINS *et al.* 2000; HOWE *et al.* 2004). POEA is supposed to take effect by increasing the membrane permeability of amphibian skin melanophores, which was observed in the African clawed frog (*Xenopus laevis*) (HEDBERG & WALLIN 2010). Also, POEA has been shown to disrupt respiratory membranes in aquatic organisms (DINEHART *et al.* 2009) and considered responsible for gill damage (RELYEA & JONES 2009; DINEHART *et al.* 2010; WILLIAMS & SEMLITSCH 2010). In concert with these findings, for instance, Roundup Original® (including POEA) was found to be 'highly toxic' to larval Pacific chorus frog

(*Pseudacris regilla*) (KING & WAGNER 2010). The 24-h LC50 (0.23 mg a.e./L) was below drinking water standards defined by the USEPA (WILLIAMS & SEMLITSCH 2010). However, also other surfactants can pose risks to tadpoles; for example, GBH with nonylphenol surfactants are also more toxic than GLY alone (TRUMBO 2005), and new GBH formulations such as Roundup OriginalMAX® and Roundup WeatherMAX® (with unknown surfactant systems) are more or less equally or even more highly toxic to tadpoles than POEA-containing GBH (RELYEA & JONES 2009; RELYEA 2011). Roundup OriginalMAX® was 'highly toxic' to tadpoles of the American bullfrog (*Lithobates catesbeianus*) and the Spring peeper (*Pseudacris crucifer*) (RELYEA & JONES 2009). In the most extreme case, exposure to Roundup WeatherMAX® at levels as low as drinking water standards (0.57 mg a.e./L) resulted in 80% mortality in tadpoles of the Gray treefrog (*Hyla versicolor*) (WILLIAMS & SEMLITSCH 2010). Overall, GBH including surfactants are known to induce oxidative stress in larvae of the neotropical toad *Rhinella arenarum* (LAJMANOVICH *et al.* 2011) and American bullfrogs (COSTA *et al.* 2008), which could be another mechanism for toxicity next to the disruption of skin melanophores or respiratory membranes (COSTA *et al.* 2008; LAJMANOVICH *et al.* 2011).

Variation in sensitivity: Some light may be shed on the intraspecific (within a species) and interspecific (between species) variation in sensitivity to GLY and GBH to better understand some varying or seemingly inconsistent outcomes. Intraspecific variation was observed for the different life stages of amphibians. Tadpoles form a rather sensitive life stage compared to terrestrial life stages (BIDWELL & GORRIE 1995; MANN & BIDWELL 1999) and embryos of the same species, apparently because the latter miss target organs, such as functional gills, or they have become insensitive (EDGINTON *et al.* 2004; HOWE *et al.* 2004). Tadpoles of later developmental stages (commonly these are expressed in Gosner stages: GOSNER 1960) were sometimes more sensitive than earlier conspecifics, which may be explained in some cases with space competition in larger tadpoles (SMITH 2001). In several cases, it could be shown that reactions were species-specific. Differences in sensitivity over anuran families have been found. The general sensitivity order to GBH of common amphibian families is: Hylidae > Ranidae > Bufonidae (KING & WAGNER 2010; WILLIAMS & SEMLITSCH 2010). Note that this order is not universal for all pesticides as, for instance, ranid frogs are supposed to be more sensitive to endosulfan than hylid frogs (HAMMOND *et al.* 2012).

Salamander larvae seem to be more resistant than those of anurans (RELYEA & JONES 2009; KING & WAGNER 2010). For example, different from tadpoles, no mortality of larval salamanders was observed after 24 h in 1 study (KING & WAGNER 2010). This could be related to the fact that carnivorous salamander larvae filtrate much less water by time and have a lower activity rate than sometimes obligately filtrating anuran larvae with higher activity during foraging (DUELLMAN & TRUEB 1986).

With regard to variation at the population level, acute toxicity tests on two spadefoot species larvae (*Spea bombifrons, S. multiplicata*), from cropland and natural grassland, indicated no significant differences when exposed to Roundup WeatherMAX® (DINEHART *et al.* 2010). Pesticide tolerance due to regular applications in cropland has not been observed. A related study showed varying sensitivities among 10 populations of Southern leopard frogs (*Lithobates sphenocephalus*) obtained from different localities which were all exposed to the same concentrations of the insecticide carbaryl (BRIDGES & SEMLITSCH 2000). However, since previous pesticide exposure or land-use around the populations was not considered, it remains unclear if the observed variations were in fact caused by local adaption to the pesticide or just due to random effects of geographic variation (BRIDGES & SEMLITSCH 2001). In addition, significant carbaryl tolerance among individuals of the same population has been found for this species and for *Hyla versicolor* and linked to genetic variations (SEMLITSCH *et al.* 2000; BRIDGES & SEMLITSCH 2001). Likewise, slightly different 96-h LC50 values (both studies with a nonrenewal design) of 2.0 mg a.e./L (HOWE *et al.* 2004) and 4.22 mg a.e./L for Roundup Original® (FUENTES *et al.* 2011) were observed for Green frog (*Lithobates clamitans*) larvae in the same developmental stage but sampled from different localities. However, many other factors, such as variation between the laboratories, may be responsible for the difference.

Effects of sublethal concentrations

Unnatural deformity rates in larvae: Just 2 studies investigated deformity rates after GBH exposure and found effects, but due to the small number the null hypothesis that GBH has no effects on deformity rates cannot be rejected (p>0.05) and further studies are required. Internal and external damages (craniofacial and mouth deformities, eye abnormalities, and bent, curved tails) were observed directly after 96-h LC50 acute toxicity testing in surviving treefrog (*Scinax nasicus*) tadpoles (LAJMANOVICH *et al.* 2003) and in Northern leopard frog (*Lithobates pipiens*) larvae when they were raised until metamorphosis (HOWE *et al.* 2004).

Endocrine disruption in larvae: One study stated endocrine effects of POEA and GBH, while another reported no effects of GLY (p>0.05). In Northern leopard frogs the thyroid axis was disrupted due to an increased thyroid hormone receptor β mRNA expression when tadpoles were exposed to Roundup Original® at 1.8 mg a.e./L and Roundup Transorb® at both 0.6 and 1.8 mg a.e./L (HOWE *et al.* 2004). Whether some GBH may affect the sexual development of frogs and thereby affect the reproductive portion of a population remains unclear. While about 20% of Northern leopard frogs developed abnormal gonads ('intersex') after exposure to POEA, Roundup Original® or Roundup Transorb®, the sex ratio was unaffected when compared to the controls (HOWE *et al.* 2004). Another study, which tested GLY but not GBH, found that gonadal steroidogenesis in *Pelophylax* kl. *esculentus* was not affected by GLY but was by another herbicide (paraquat) (QUASSINTI *et al.* 2009).

Effects on development: Here, 6 out of 6 studies found effects (p<0.01) at least on some of the studied species. Precipitated (*Rana cascadae*) (CAUBLE & WAGNER 2005) or delayed metamorphosis (*L. pipiens*, *Anaxyrus americanus*) (HOWE *et al.* 2004; WILLIAMS & SEMLITSCH 2010) have been observed, apparently depending on the studied species. Delayed time to metamorphosis can be caused by disruption of the thyroid axis (see *Endocrine disruption in larvae*) or energy consumptive detoxification processes in tadpoles. Also, tadpoles of some species are able to metamorphose earlier under stress, for example, when predators are present or when the pond is drying out (VAN BUSKIRK & RELYEA 1998; RELYEA 2004a). In a field experiment which studied the effect of the GBH formulation Accord®, with nonylphenol etoxylate as surfactant, larvae of Northern leopard frogs increased in size, while larval Tiger salamanders (*Ambystoma tigrinum*) decreased in a density-dependent manner (BRODMAN *et al.* 2010). Another study found that Roundup OriginalMAX® induced morphological changes in tadpoles similar to the adaptations induced by predator cues suggesting that the herbicide activates developmental pathways used for antipredator responses (RELYEA 2012).

Inhibition of specific enzymes: Only 1 study investigated inhibition of certain enzymes and observed effects after short exposure (24-48 h). Some of the tested GBH inhibited enzymes involved in neurotransmission and detoxification (esterases) at sublethal concentrations in the test species *Rhinella arenarum* (LAJMANOVICH *et al.* 2011). Inhibition of these enzymes put the animals under general stress and reduced their individual fitness. Similar to acute toxicity, the responses of the test species remarkably differed with the tested GBH (LAJMANOVICH *et al.* 2011). It is notable that SAMSEL & SENEFF (2013) compiled evidence that GLY inhibits cytochrome P450 enzymes in human liver cells and rats. Like esterases, cytochrome P450 enzymes are involved in detoxification of xenobiotics; the authors conclude that GLY enhances the damaging effect of environmental toxins. Since cytochrome P450 enzymes are widespread in nature, their possible inhibition by GLY should be tested in amphibians as well because that could explain synergistic effects of GLY with other pesticides.

Genotoxic effects: Both retrieved studies concerning this endpoint found genotoxic effects (but $p>0.05$, see *Unnatural deformity rates in larvae*). Using the standard methods 'comet assay' and 'micronucleus test', it was found that some GBH have a genotoxic and mutagenic potential on tadpoles of the American bullfrog (CLEMENTS *et al.* 1997) and on adults of the neotropical *Odontophrynus cordobae* and *Rhinella arenarum* (BOSCH *et al.* 2011). Damage of this kind could negatively affect individuals as well as their offspring. Out of 5 tested herbicides, especially Roundup Original® (together with a metalochlor formulation) had the greatest impact on tadpoles of the American bullfrog (CLEMENTS *et al.* 1997).

Effects on embryos: Four out of 5 studies found effects on embryos ($p<0.05$), but only 1 directly stated teratogenicity – increased mortality and malformation rates (PAGANELLI *et al.* 2010). One study observed increased malformation rates only at concentrations above the 96-h LC50 (PERKINS *et al.* 2000), and similarly, 2 other studies reported effects on body indices (MANN & BIDWELL 2000; ORTIZ-SANTALIESTRA *et al.* 2011). Applying the 'Frog Embryo Teratogenesis Assay – Xenopus' (FETAX), no significant increase of malformation in tadpoles was observed at concentrations of Roundup Original® and Rodeo® that were below 96-h LC50 for embryos. The surfactant POEA alone was more toxic (96-h LC50 6.8 mg a.e./L) than Roundup Original® containing POEA, which was 700 times more toxic than Rodeo® lacking a tallowamine surfactant (96-h LC50 9.3 mg a.e./L and over 7,000 mg a.e./L, respectively) (PERKINS *et al.* 2000). Using a different study design than the standardized FETAX, teratogenic effects of both GLY and GBH on *X. laevis* have been reported (PAGANELLI *et al.* 2010). In that study, embryos showed an increase of head defects and craniofacial malformations, but when they were exposed to a 1/5000 dilution of Roundup Classic® equaling 72 mg a.e./L (*i.e.*, nearly the eightfold LC50 found in PERKINS *et al.* [2000]) or when GLY was directly injected (360 pg and 500 pg). That study (PAGANELLI *et al.* 2010) was followed by strong replies about the pertinence of study designs (especially high doses and injection) and conflicting interests (BVL 2010a). In another study, late embryos of *X. laevis* that were in the period of organ morphogenesis were exposed to different concentrations of Roundup Original® (0.25 mg, 0.5 mg, 1 mg, and 5 mg of formulation per liter) for only 2 d. No significant mortality or malformation rate was observed (LENKOWSKI *et al.* 2010). When GBH nonylphenol surfactants were tested, they did not produce significant teratogenic effects in anuran embryos but inhibited growth (MANN & BIDWELL 2000). Roundup® (exact formulation unknown) had no impact on survival or malformation rate in an experiment with embryonic stages of the Gold-striped salamander (*Chioglossa lusitanica*), but exposed embryos had a larger mean size at hatching than the control group (ORTIZ-SANTALIESTRA *et al.* 2011). One may postulate that the observed increase in body size is related to an impact of the GBH on the thyroid hormonal balance as observed in other studies (GUTLEB *et al.* 2000).

Behavioral effects: One study stated effects and one did not ($p>0.05$). Gray treefrogs similarly avoided artificial breeding ponds either containing predatory fish or GBH (TAKAHASHI 2007). It is intriguing to suggest that the treefrogs may both perceive external stimuli and trigger similar reactions as already suggested for morphological adaptations in tadpoles (see *Effects on development*). However, the sample size in this study was rather small. In another study, no effects on site selection in European frog and newt species were observed, for either GLY or GBH (WAGNER & LÖTTERS 2013).

Interactions with abiotic and biotic amphibian stressors

Some authors have tried to simulate aquatic communities in which amphibian larvae (mainly tadpoles) occur (RELYEA 2005a, c; RELYEA 2009; JONES *et al.* 2010, 2011). In particular, the findings derived from such mesocosm studies have provided information on how applied GBH can interact with other factors, including stressors that can be present in larval amphibian habitats. Others have tested the effect of GBH treatment and 1 additional stressor under laboratory conditions (CHEN *et al.* 2004; EDGINTON *et al.* 2004; RELYEA 2004b).

Abiotic interactions: Seven out of eight studies stated effects of abiotic costressors on considered endpoints ($p<0.01$). Several mesocosm studies which tested GLY or GBH included sediments in their setting. It is a matter of discussion whether sediments interfere with the outcome due to adsorption of the test substances. While 1 study suggests just that (BERNAL *et al.* 2009a), another denies it and assumes that the primary, acute toxic effect of the substances takes place before they adsorb to the sediment (RELYEA 2005a). In 1 mesocosm study, mixtures of Roundup Original® and other pesticides (4 other herbicides and 5 insecticides) at sublethal concentrations (10 µg a.e./L of each pesticide), affected larval survival due to indirect effects (RELYEA 2009). Each pesticide was tested singly and in combination with other pesticides on tadpoles of 2 North American species (*Lithobates pipiens, Hyla versicolor*). Insecticide-induced decline of zooplankton, which is the assumed primary acute effect, led to an increase of phytoplankton and – because of increased shadowing – a decrease of periphyton, which is consumed by tadpoles. Hence, insecticides (particularly endosulfan and diazinon) had the largest impact on tadpole survival and single GBH effects did not stand out (RELYEA 2009). Another study investigated the impact of pesticide mixtures on tadpoles of North American anurans (*Lithobates pipiens, L. clamitans, L. catesbeianus, Anayxrus americanus, Hyla versicolor*) under laboratory conditions (RELYEA 2004b). Each pesticide was tested alone (*i.e.*, Roundup Original® and 3 insecticides) at 1 mg a.i./L and 2 mg a.i./L, respectively, and in pairwise combinations (1 mg a.i./L of each pesticide). Larval growth was affected in nearly all cases. At low concentrations, pesticides alone had no negative impact on tadpole survival. At high concentrations, all pesticides tested – except for carbaryl – caused significant tadpole mortality. Pesticide mixtures occasionally affected larvae more than single pesticide treatments, but the impact of pesticide mixtures on survival never exceeded that of the single pesticides at 2 mg a.i./L. Therefore, growth and survival can be predicted from the total concentration of the tested pesticides (RELYEA 2004b). With regard to Caudata, mixtures of Roundup Plus® and a fertilizer had no effect on salamander embryos (ORTIZ-SANTALIESTRA *et al.* 2011). Other studies showed that mortal effects of GBH including the surfactant POEA increased at higher pH levels. This may be related to the circumstance that the nonionized form of POEA, which is present at higher pH levels, readily accumulates on the gills of tadpoles (CHEN *et al.* 2004; EDGINTON *et al.* 2004) Additional sublethal ultraviolet-B radiation to GLY or Roundup® exposure significantly reduced survival of *L. clamitans* tadpoles (PUGLIS & BOONE 2011).

Biotic interactions: Eight out of 12 studies stated effects of biotic costressors on considered endpoints ($p<0.01$). An increase of trematode infection in GLY-treated (3.7 mg a.e./L) tadpoles has been found (ROHR *et al.* 2008), suggesting immunosuppressive effects; but no synergistic effects of GBH exposure and zoospores of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) have been observed (EDGE *et al.* 2011, 2013; PAETOW *et al.* 2012). This is the agent of the dangerous emerging amphibian disease chytridiomycosis, hypothetically responsible for population declines in many species (MENDELSON *et al.* 2006; STUART *et al.* 2008). There is also a study reporting that GBH, here Roundup WeatherMAX® at 2.89 mg a.e./L, reduces mortality of Wood frog (*Lithobates sylvaticus*) tadpoles caused by these zoospores (GAHL *et al.* 2011). A plausible explanation is that treatment with Roundup

WeatherMAX® affected the pathogen more than the frogs (GAHL *et al.* 2011), which is in line with reports about the inhibitory effect of GLY on fungi (DILL *et al.* 2010). It is surprising that in this study GBH on its own had no effect on the survival of tadpoles (the experiment lasted until metamorphosis), which is not in line with 96-h LC50 values (nonrenewal) determined for Roundup WeatherMAX® (1.33-3.26 mg a.e./L) in 6 other North American anuran species (FUENTES *et al.* 2011). However, this could be related to the aforementioned variation in sensitivity or differences between the laboratories.

Predator cues and competition can enhance the toxicity of contaminants including pesticides (RELYEA 2005b; BRODMAN *et al.* 2010; JONES *et al.* 2011). Predator cues can make pesticides significantly more toxic to tadpoles (up to 46 times for carbaryl) (RELYEA & MILLS 2001; RELYEA 2003). However, only some species were affected by the presence of predator cues when exposed to GBH (RELYEA 2005b). Experiments have demonstrated that intraspecific competition among individuals of the American bullfrogs not only reduced their growth, but also could make individual tadpoles more susceptible to GBH (JONES *et al.* 2011). In another study, North American treefrogs (*Hyla versicolor, H. chrysoscelis*) avoided breeding ponds containing either predatory fish or GBH (TAKAHASHI 2007).

Discussion

Acute toxicity

The literature survey has revealed that the sensitivity of amphibians to GBH is formulation, species and life-stage-specific (surfactants > GBH > GLY; Anura > Urodela; Hylidae > Ranidae > Bufonidae, larvae > embryos > juveniles and adults). GBH with POEA or other tallowamine surfactants are ‘moderately toxic’ or even ‘highly toxic’ to larvae, but so are some new GBH formulations that contain unspecified surfactants. Direct overspraying of terrestrial life stages with GBH can pose risk, the actual harm depending on other factors including formulation, vegetation buffers, etc. Because the potential impact of most pesticides on terrestrial life stages is still unknown (BRÜHL *et al.* 2011), a comparison of the application of GBH with other herbicides is not possible yet and further research is needed. Overall, GBH can be classified as ‘moderately toxic’ (Fig. 1) and GLY as ‘slightly toxic’ to ‘practically nontoxic’ to amphibian larvae. A general classification of the toxicity of GBH or GLY for embryos and terrestrial life stages seems not to be reasonable due to a lack of studies.

Effects of sublethal concentrations

Results of different studies indicate that also chronic and delayed effects are formulation and species-specific. For example, GBH exposure precipitated metamorphosis in *R. cascadae* (CAUBLE & WAGNER 2005), delayed it in *L. pipiens* and *A. americanus*, and hardly affected it in other anuran species (HOWE *et al.* 2004; WILLIAMS & SEMLITSCH 2010). Although there has been more research on toxicological effects of GLY and GBH on amphibians compared to other pesticides, studies on effects of sublethal exposure do not allow conclusions about most considered endpoints due to a lack of data. Apparently, developmental parameters of larvae (*e.g.*, body indices at metamorphosis, time to metamorphosis) are the most sensitive endpoints to study, in so far as this is known. This corresponds with the finding that the aquatic larval stage is also the most sensitive life stage.

The occurrence of abnormal gonads (testicular oocytes) in the study by HOWE *et al.* (2004) has recently been attributed to normal-development hermaphroditism in leopard frogs (GUBBINS *et al.* 2013). There could also be variation in response to endocrine disrupters based in life history of the species exposed (STORRS & SEMLITSCH 2008), and no definitive answer could be given because of the small number of studies.

It remains also unclear if GLY and GBH have teratogenic effects and further studies are desirable. In this context, the extent to which *X. laevis* represents the response of other taxa will be briefly discussed. Some authors question if *X. laevis* is sensitive enough to be a sentinel species. Although husbandry conditions can affect the sensitivity of this species (KLOAS *et al.* 2009), it is a good sentinel species for, among other things, endocrine responses (SOLOMON *et al.* 2008; ROHR & MCCOY 2010). Several studies on acute and developmental toxicity compared responses of *X. laevis* and other, phylogenetically distinct anuran species to different contaminants. The results showed that *X. laevis* is among the more sensitive and often the most sensitive of the species tested (SCHUYTEMA *et al.* 1991; SCHUYTEMA & NEBEKER 1998, 1999; LENKOWSKI *et al.* 2010). When responses of embryos and early larvae of several amphibian species including *X. laevis* to the GBH Vision® were compared at varying pH values, there was no clear trend (EDGINTON *et al.* 2004); but in a different study agricultural surfactants narcotized (EC50) tadpoles of *X. laevis* more than larvae of other species (MANN & BIDWELL 2001). In general, *X. laevis* is among the most sensitive amphibian species and serves as good sentinel species in amphibian risk assessments. However, it cannot be denied that some amphibian species are more sensitive to certain contaminants. They can be taken into account with sufficient safety factors (see *Comparison between standard test organisms and amphibians*).

Interactions with abiotic and biotic amphibian stressors

Co-stressors mainly enhance the impact of GBH; therefore, GBH concentrations that have no effects when applied singly in laboratory studies may become the opposite in the wild where costressors such as predators are commonly present (SIH *et al.* 2004). However, most of the studies with costressors used relatively high concentrations of GBH. It is interesting that none of the 4 studies found adverse interactions between GBH exposure and chytrid infection.

Comparison between standard test organisms and amphibians

At this point, it seems most important to compare the responses of standard test organisms used in risk assessment with those of amphibians. The main question is if amphibians are more sensitive to GLY and GBH than standard test organisms or not. With regard to acute toxic effects, LC50 values can be compared, but chronic effect testing of GLY and GBH exposure on amphibians mainly considered endpoints such as ‘time to metamorphosis’ or ‘size at metamorphosis’. If no-observed-effect concentrations (NOEC) for amphibians were available (*e.g.*, see http://cfpub.epa.gov/ecotox/), results were calculated from differing study designs such as mesocosm experiments with costressors. Hence, a comparison of chronic effects even among amphibians is not possible yet; in terms of the comparison between amphibians and standard test organisms, we are restricted to acute toxicity.

Laboratory studies on official aquatic test organisms have revealed that GLY isopropylamine salt was ‘practically nontoxic’, technical-grade GLY acid was ‘practically nontoxic’ to ‘slightly toxic’ and Roundup Original® was ‘moderately toxic’ to them (DILL *et al.* 2010). Pesticide regulation practice in Europe and the USA consider safety factors of 10 to 100 – depending on studied species and endpoints (http://www.epa.gov/oppfead1/trac/science/fqpa_resp.pdf, http://ec.europa.eu/enterprise/epaa/3_events/3_3_workshops/full-report-tox-workshop-september-2012.pdf) and derived toxicity to exposure ratio values (meaning endpoint/exposition) (http://www.oecd.org/chemicalsafety/agriculturalpesticidesandbiocides/1944146.pdf). With regard to their allowed environmental concentration (*e.g.*, 5 µg a.e./L with 5 m buffer strip for

Germany: BVL [2010a]), risk assessment of pesticides that is based on ecotoxicological endpoints gained from tests with larval teleost fish (which also have a permeable skin) and aquatic invertebrates will most likely cover acute toxic effects on amphibian larvae including differences in the sensitivity of amphibian species (one exception would be the North American *Pseudacris regilla*: 24-h LC50=0.23 mg a.e./L [KING & WAGNER 2010], but whether similarly sensitive species occur in Germany is unknown). These findings conform to the results of a study that compared several pesticide endpoints of amphibians with those of other aquatic organisms (ALDRICH 2009). Even the presence of costressors will be covered in most cases; but, for example, the lowest LC50 value in a study on effects of Roundup® in combination with predatory stress was 0.41 mg a.e./L for Wood frogs (RELYEA 2004b). This value is just outside the 100fold safety factor (5 µg a.e./L → 4 µg a.e./L).

With regard to terrestrial lifestages of amphibians, in only 1 amphibian study (MCCOMB *et al.* 2008) was GLY isopropylamine salt administered intraperitoneally. The lowest 96-h LD50 value was 1170 mg a.e./L. The lowest 192-h LD50 value for the same substance to the bird species *Colinus virginianus* was >3851 mg a.e./kg body weight (http://cfpub.epa.gov/ecotox/), while the 120-h LD50 value of GLY acid to the same species was >4971 mg a.e/kg diet (DILL *et al.* 2010). Likewise, a single dose of GLY acid to *Rattus norvegicus* resulted in a LD50 value of 4275 mg a.e./kg body weight (DILL *et al.* 2010). Since all values are in the same order of magnitude, mammals and birds apparently serve as proxies for amphibians' risk with regard to oral administration of the active ingredient; however, studies on the toxicity of orally administered GBH to amphibians are missing.

However, as already mentioned, amphibians seem to be at higher risk due to dermal uptake that, in the case of GLY, occurs 26 times faster for amphibians than for mammals (QUARANTA *et al.* 2009; BRÜHL *et al.* 2013). Therefore, studies are required which test this exposure route, for example, by directly applying GBH or GLY on amphibians (or their skin, see QUARANTA *et al.* [2009]) or by incubating them in contaminated water. For this purpose, mammals and birds do not seem to suit and we suggest including at least 2 amphibian species from different families in standardized test batteries to study dermal administration.

Contribution to population/ species decline

The main question is if GLY and GBH use affects natural amphibian populations. Herbicide use is 1 among many partial causes of ongoing global amphibian declines (BOONE *et al.*

2007). However, its exact role remains difficult to assess as 1) field data remain sparse (DAVIDSON *et al.* 2001; DAVIDSON *et al.* 2002; DAVIDSON 2004) and 2) abnormal population changes have been suggested to often result from multiple interacting causes (COLLINS & STORFER 2003; MENDELSON *et al.* 2006; STUART *et al.* 2008). Hence, no definitive answer can be given with regard to the past, current, or future contribution of GBH as well as other environmental contaminants. In the following, some aspects are discussed regarding whether GBH use may be a relevant regional contributor to amphibian decline.

With regard to temporality, the increasing use of GBH cannot be made responsible for most amphibian population and species losses during the last decades. The majority of declines in developed countries occurred in the 1960s to 1970s (HOULAHAN *et al.* 2000), partly due to the intensification of agriculture in general. Although GBH are currently distributed in these countries, GLY marketing only started in 1974 (DILL *et al.* 2010). A new wave of dramatic declines and extinctions of amphibians has been witnessed over the last 3 decades (MENDELSON *et al.* 2006), but these have largely taken place in pristine and remote areas in the tropics. GBH use as a reason can be ruled out here because other potential causes have been identified (*e.g.*, habitat destruction and emerging infectious diseases: LA MARCA *et al.* [2005]; STUART *et al.* [2008]). Thus, there is no temporal evidence of any association between GBH use and observed amphibian declines.

With regard to concentration response relation, most laboratory and mesocosm studies state effects in a dose-response manner (see *Results*). There is consistency in studies that reported adverse effects of some GBH (especially those with tallowamine surfactants like POEA) on amphibians at concentrations that can occur in the environment (see Table 1), but also that other less harmful GBH (*e.g.*, which are labeled for aquatic use) will probably not affect individuals in normal-use scenarios. The formulation used and the site-specific application practice make the difference.

It is important to consider each life stage of amphibians separately in risk assessment. For adults, it may have fatal consequences when applications of certain GBH coincide with migration activities, for example, when the main reproductive part of a population crosses fields during seasonal migrations to breeding sites and would be directly oversprayed (RELYEA 2005a), but this holds true for other pesticides as well (BRÜHL *et al.* 2013). That can also happen during the daytime when pesticides are applied (as it is the case in ‘explosive breeding’ European amphibians: WELLS [1977]). Such mass mortalities of adults due to tilling have already been observed and studied in the field (DÜRR *et al.* 1999), and one can postulate that herbicide applications, that replace mechanical weed management in no-tillage farming, can have similar effects. Nondirected migration of newly metamorphosed juveniles that emerged from water bodies near agricultural land has also been observed, leading them onto fields (DÜRR *et al.* 1999) (threat of direct overspraying, *e.g.*, in winter grain cultivation). However, it will not matter for an amphibian population that persists in an agrarian landscape if migrating individuals suffer ‘mechanical’ or ‘chemical death’. Most studies used tadpoles and some described adverse (mainly chronic) effects at environmentally relevant concentrations. Additional effects of costressors in the natural environment can enhance impacts of GBH concentrations. It is important to note that the majority of anuran species follow a reproductive strategy of 'overproduction' enduring high mortality rates of their offspring (SCHMIDT 2004), and that, therefore, (subjectively) high mortality or failure rates in larvae may not substantially affect population viability (VEITH & VIERTEL 1993; FORBES & CALOW 2002). However, early metamorphosis is accompanied by limited body condition (size, mass), resulting in reduced individual fitness (*e.g.*, decreased overwinter survival and reproductive potential and prolonged time to first reproduction) (SMITH 1987, 2001; SEMLITSCH *et al.* 1988; GOATER 1994; ALTWEGG & REYER 2003). Delayed metamorphosis lengthens the time larvae are vulnerable to aquatic predators and may increase mortality rates in species using ephemeral ponds (common in anurans), as these habitats are more likely to dryout towards the summer or in dry periods (BOONE *et al.* 2007). Effects of both precipitated and delayed metamorphosis on population viability are poorly understood yet (DÜRR *et al.* 1999).

Many of the above-named potential threats to different amphibian life stages do not exclusively apply to GBH but to most other pesticides as well. However, unique to GBH – and other nonselective herbicides – is their application during the whole growing season. GBH can be used from the time before sowing in no-tillage farming until harvest (desiccation of several conventional crops: DILL *et al.* [2010]). Furthermore, GBH are applied later in HR-systems compared to conventional cultivations because they allow eliminating weeds post-emergence of the HR crops (BVL 2010b). Hence, some amphibian species may be multiply affected by GBH applications during the year. Although comprehensive official data are missing, it can be inferred that GM soybean has driven agricultural expansion and land use change in Argentina and other South American countries (PENGUE 2005) meaning that the complementary GBH was applied in former forest land and other natural habitats. With the advent and adoption of HR crops that additionally tolerate unfavorable conditions (*e.g.*, soil pH or drought), GBH might extend even further into natural habitats.

Conclusion

To study the contribution of GBH (and any other pesticide) use to observed amphibian population declines, a species- and site-specific evaluation has to be conducted. We see the need to include population viability analyses into amphibian risk assessment. These have been successfully used with regard to similar questions, for instance, impacts of elevated ultraviolet-B radiation on amphibian populations (VONESH & DE LA CRUZ 2000, 2002). Therefore, field data of both amphibian populations in the agrarian landscape and contamination with GBH and other pesticides are needed. While this involves cost and time-intensive fieldwork, it cannot always guarantee a general answer as demonstrated in case of the European treefrog (*Hyla arborea*). This species is disappearing from many agrarian landscapes where it was previously common, although when terrestrial and aquatic habitats are still present (GLANDT 2004). Treefrogs are the anuran family most sensitive to GBH, but their disappearance cannot be causatively linked to an increasing GBH or other pesticide use. The same holds for the observed disappearance of other sensitive species in agrarian landscapes. While GBH use is increasing, farming has been intensified in general and treefrog populations are affected by a multitude of other factors (such as landscape fragmentation) (GLANDT 2004). Another case is the Argentinean anuran fauna of agricultural sites with intensive cultivation of HR soybeans. Individuals show significant enzymatic alterations and reduced body size (BRODEUR *et al.* 2011), but it remains unclear how pesticides are applied locally and to what proportion GBH or other pesticides (*e.g.*, insecticides) can be causatively linked to poor body conditions.

In general, more field work and modeling are required on how failure rates of different amphibian life stages contribute to the survival of populations in the agrarian landscape. Without such work, amphibian population declines cannot causatively be linked to GBH or other pesticide use. However, this should not distract from the possibility that GBH overapplications or missing buffer strips may indeed contribute to current or future damage to local amphibian populations. Illegal over-applications of pesticides in general and ignorance of buffer strips are well known, for instance, in Germany (http://www.umweltdaten.de/publikationen/fpdf-l/3566.pdf). Stricter controls of farmers may be recommended.

As is the case in the European Union, animal species listed in Annex IV(a) of the Habitats Directive 92/43/EEC are strictly protected within their natural range and any deterioration or destruction of their breeding sites or resting places is prohibited by Article 12(1)(d). Many amphibians that inhabit agricultural landscapes are listed in Annex IV(a). Only this requires further monitoring action of both GBH contamination and amphibian populations in agrarian landscapes. Amphibian monitoring, as proposed for HR crop cultivation in some European countries (BÖLL *et al.* 2013), could help in recognizing population changes related to GBH.

We see the need to expand future research in 3 main areas: 1) filling basic knowledge gaps through studies with comparable design and modeling addressing especially chronic and delayed effects of GBH and added substances; 2) long-term monitoring of GBH in the environment and of free-living amphibians, aiming at the detection of effects on individuals and especially abnormal local population changes; and 3) an ongoing analysis of information and risk assessment (especially of new substances to come and costressors) in the context of worldwide amphibian decline and extinction. Research in these areas is well in line with the recommendations formulated under International Union for Conservation of Nature's 'Amphibian Conservation Action Plan' (BOONE *et al.* 2007), which in general points towards further research on environmental contaminants.

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Evaluating the risk of pesticide exposure for amphibian species listed in Annex II of the European Union Habitats Directive

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Highlights

- We evaluated the pesticide exposure risk for protected European amphibian species.
- Species at high risk are usually not threatened within their entire territories.
- Most globally threatened and European priority species are at low risk.
- Exposure risk within conservation areas differs on a national scale.
- Species and site-specific management plans should consider monitoring of habitat contamination.

Abstract

Environmental contaminants like pesticides concern amphibian conservationists. For many European amphibian species, special areas of conservation were created as they are listed in Annex II of the EU Habitats Directive. Agriculture is not prohibited within these conservation areas. In the present study, a risk evaluation at the European level was conducted to identify proportions of land use with regular pesticide applications within the conservation areas and the specific risk of pesticide exposure depending on the species' biology and ecology. The proportion of agricultural land use and the risk of habitat and individual contamination differ among species but also at national scale. Nearly all species with high risk of habitat pesticide exposure are not threatened within their entire territories and Europe. Conversely, most globally threatened and European priority species are at a lower exposure risk in their habitats – with the exceptions *Rana latastei*, *Pelobates fuscus insubricus*, *Triturus dobrogicus* and *Discoglossus jeanneae*. In the conservation areas for these species, Habitat Directive management plans need to consider monitoring of habitat contamination with pesticides. Such a monitoring should also be conducted in conservation areas for amphibians that seem to be not threatened yet but are at high exposure risk, *e.g.*, *Bombina bombina*. Monitoring and conservation action should also take place site-specifically to avoid national or regional loss of amphibian biodiversity. Overall, intensive use of agrochemicals and recent land use changes have the potential to be a serious threat for amphibian species, which can be found within cultivated areas – regardless of their current IUCN status.

Keywords: IUCN, Red list, management plan, insecticide, herbicide, fungicide

Introduction

Amphibians are declining worldwide at alarming rates, with more than one third of all ca. 7,000 species being threatened with extinction (HOULAHAN *et al.* 2000; MENDELSON *et al.* 2006; STUART *et al.* 2008). Various causes and their interactions are discussed to be responsible (COLLINS & STORFER 2003). They remarkably differ at continental and regional scales (GASCON *et al.* 2007), and in 'industrialized' regions, such as North America and Central Europe, environmental contamination is listed among important factors (BOONE *et al.* 2007; HAYES *et al.* 2006).

The increasing use of pesticides is worrying amphibian conservationists (RELYEA 2011). It is known to affect different life-stages. Up to 100% mortality has been shown for juvenile anuran amphibians (*i.e.*, frogs and toads) when over-sprayed with fungicides or herbicides at approved application rates (RELYEA 2005; BELDEN *et al.* 2010; BRÜHL *et al.* 2013). In frogs and toads that inhabit intensively used agricultural areas, MCCOY *et al.* (2008) have shown alterations of gonadal form and functions, and LAJMANOVICH *et al.* (2010) found inhibition of certain enzymes involved in neurotransmission and detoxification. For aquatic larvae, acute, chronic, and delayed effects have been demonstrated when exposed to environmentally relevant pesticide concentrations (HOWE *et al.* 2004; HAYES *et al.* 2006). Likewise, pesticides are proposed to affect amphibians *in ovo* (ORTON & ROUTLEDGE 2011).

All life-stages of amphibians can be exposed to pesticides, for instance, by way of drift (DAVIDSON *et al.* 2002; DAVIDSON 2004), wind erosion and run-off of contaminated soil (BROWNER 1994), or direct over-spraying, especially, when animals pass agricultural land during their annual migrations (RELYEA 2005; DINEHART *et al.* 2009; LENHARDT *et al.* 2013). Exposure risk for amphibians is greater compared to most other animal groups because amphibians take up xenobiotics several times faster over their highly permeable skin (QUARANTA *et al.* 2009). Indirect exposure through contaminated food (MCCOMB *et al.* 2008) or contaminated soil and plant material (BRÜHL *et al.* 2011) may also be important routes of exposure. In addition, indirect effects are reported in some cases, for instance, avoidance of contaminated breeding ponds leading to a loss of suitable surface waters (TAKAHASHI 2007; VONESH & BUCK 2007; but see VONESH & KRAUS 2009; WAGNER & LÖTTERS 2013). From these points it is obvious that pesticide use can affect amphibian health (MANN *et al.* 2003, 2009; BRÜHL *et al.* 2011). However, its contribution to population or species decline remains unclear due to the current lack of data and uncertainties in identifying causative agents of decline (SCHMIDT 2004; WAGNER *et al.* 2013).

The Habitats Directive of the European Union (EU 1992) is a major part of the legal framework of the Natura 2000 network of conservation areas. Although it was criticized, among others, for lack of flexibility concerning fixed lists of protected species (HOCHKIRCH *et al.* 2013) or insufficient consideration of optimal site designation (GASTON *et al.* 2008), this network is considered as one of the largest and most important conservation networks worldwide (LOCKWOOD 2006). Annex II of the Habitats Directive lists species which are of "… community interest whose conservation requires the designation of special areas of conservation (= SAC)" (EU 1992). With regard to amphibians, 22 species and 3 subspecies are listed in Annex II. Four are even 'priority species' of the Natura 2000 network, which requires an enhanced protection status (EU 1992; Table 1).

Numerous problems in conservation areas arise from land use conflicts (JETZ *et al.* 2007). Today, massive land use changes can be observed in Europe, for instance, as a result of the growing impact of 'energy crops' (*e.g.*, corn as a biofuel source), leading to an increasing demand for areas under crop (FARGIONE *et al.* 2010). In Europe there is a trend to grow 'energy crops' on previously uncultivated land such as fallow land, degraded land, former mining areas etc. (DAUBER *et al.* 2013) which may represent crucial habitats for amphibian survival (*e.g.*, BÖHME *et al.* 1999). In addition to habitat destruction, this transformation process also increases the risk of pesticide exposure to wildlife. In the future, also cultivation of genetically engineered crops – which are created to stand adverse abiotic conditions like too high or low soil pH – is expected to expand in previously non-arable European areas, as it can already be observed in other parts of the world (*e.g.*, Argentina: PENGUE 2005). Agricultural land use does not stop at the border of SAC because (at defined conditions) land use within these conservation areas is still possible (EU 1992).

For the aforementioned reasons – namely conservation requirements for the protection of amphibian diversity and potential threats to amphibians due to environmental contaminants – information is required to what extent land use practices with regular pesticide applications are likely to affect amphibians within their SAC. Therefore, we conducted a risk evaluation at the European level to identify the proportions of land use with regular pesticide applications within SAC that were created for Annex II amphibians and the risk of pesticide exposure for them depending on their species-specific biology.

Tab. 1: Categories under the IUCN Red List of Threatened Species, 'land use with regular pesticide applications' (LPA) within 'special areas of conservation' (SAC), 'habitat exposure indec' (HEI) and 'pesticide risk factors' (PRF) of Annex II amphibians.

Note that global IUCN status is equivalent to the European Red List status by TEMPLE & COX (2009). Above-average PRF are written in bold.

IUCN status	LPA[a] within SAC	HEI	PRF
	Critically Endangered		
* *Salamandra atra aurorae*	0.72 %	0	0
	Endangered		
Hydromantes supramontis	3.02 %	0	0
	Vulnerable		
* *Alytes muletensis*	1.97 %	1	0.01
Chioglossa lusitanica	11.33 %	1	0.03
Hydromantes flavus	7.36 %	0	0
Hydromantes genei	4.61 %	1	0.01
* *Proteus anguinus*	8.76 %	1	0.02
Rana latastei	31.79 %	3	**0.24**
	Near Threatened		
Discoglossus montalentii	0.73 %	0	0
Hydromantes ambrosii	4.20 %	0	0
Hydromantes imperialis	3.04 %	1	0.01
Hydromantes strinatii	1.11 %	0	0
Triturus dobrogicus	22.73 %	3	**0.17**

IUCN status	LPA[a] within SAC	HEI	PRF
	Least Concern		
Bombina bombina	21.61 %	4	**0.22**
Bombina variegata	12.98 %	2	0.07
Discoglossus galganoi (including *D. jeanneae*) [b]	24.86 %	3	**0.19**
Discoglossus sardus	13.54 %	0	0.03
Lissotriton vulgaris ampelensis [c]	5.95 %	4	0.06
Lissotriton montandoni	5.31 %	2	0.03
** Pelobates fuscus insubricus* [c]	40.04 %	4	**0.40**
Salamandrina terdigitata	10.87 %	1	0.03
Triturus carnifex	18.47 %	3	**0.14**
Triturus cristatus	17.31 %	3	**0.13**
Triturus karelinii	19.12 %	3	**0.14**

* =priority species

[a] = LPA = Land use with regular pesticide applications according to its CORINE land cover classes

[b] = *D. jeanneae* is listed as Near Threatened

[c] = No specific IUCN validation for this subspecies

Material and methods

Land use with regular pesticide applications within the SAC

We calculated the proportion of 'land use with regular pesticide applications' (LPA) within SAC that were created for Annex II amphibian species using ArcMap 10 (Esri®) and the latest version of the European CORINE (Coordination of Information on the Environment) land cover data. CORINE data and those for Natura 2000 sites and species were obtained from the European Environmental Agency (http://eea.europa.eu). In the CORINE project, mapping of the land cover was performed on the basis of satellite remote sensing images on a scale of 1:100,000. Agricultural land cover classes (under the CORINE-Label "Agricultural areas"), which reflect areas where pesticides are regularly applied, were chosen, namely CORINE land cover classes 211 ("non-irrigated arable land"), 212 ("permanently irrigated land"), 213 ("rice fields"), 221 ("vineyards"), 222 ("fruit trees and berry plantations"), 223 ("olive groves"), 241 ("annual crops associated with permanent crops"), 242 ("complex cultivation patterns"), 243 ("land principally occupied by agriculture with significant areas of natural vegetation") and 244 ("agro-forestry areas"). Cultivation and pesticide use practices differ between and in these classes (often annually), but more detailed information is not available for the entire EU. On intensively used hay meadows, pesticides are regularly applied, but we excluded land cover class 231 ("pastures") because it is not possible to distinguish between this type of grassland and real pastures. Conversely, parts of the European agricultural area are organic (see discussion). No actual land cover data were available for Greece and the UK, so that this country was excluded from the evaluation.

'Habitat exposure indices' and 'pesticide risk factors'

Since life history aspects of the considered amphibian species remarkably differ, we created a 'habitat exposure index' (HEI) for each species reflecting their potential risk of pesticide exposure on the basis of literature information and presence/absence data. Three 'evaluation factors' (EF) for exposure risk were considered to define the HEI.

Eventually, the HEI for each species together with the proportion of agricultural land use defined the species' 'pesticide risk factor' (PRF).

'Evaluation factor' for aquatic and terrestrial habitat exposure risk (EF 1)

Exposure of habitats can occur via pesticide drift during applications, wind erosion of contaminated soil, run-off, or direct over-spraying. Indirect contact of animals with pesticides can occur via contact with contaminated soil/plant material or the food chain. The question was if exposure as well as indirect contact can be expected because it is likely that habitats are situated within or next to agricultural land. For example, the Fire-bellied toad (*Bombina bombina*) often breeds in small, open water bodies in cultivated landscapes (NÖLLERT & NÖLLERT 1992) where pesticide residues may easily accumulate (MANN *et al.* 2003). The Common spadefoot, *Pelobates fuscus,* is often known to inhabit fields (BÖHME *et al.* 1999). Conversely, other species are probably at 'intermediate' habitat exposure risks. For example, *Triturus cristatus* (Northern crested newt) can be found in both agricultural areas and in habitats with lower risk of pesticide contamination like quarries or former mining landscapes (BÖHME *et al.* 1999). Other species predominantly live in remote habitats mainly away from cultivated areas, for instance, the Golden salamander (*Salamandra atra aurorae*) or the Corsican painted frog (*Discoglossus montalentii*), or even live subterraneously during most of their life, like Web-toed salamanders (*Hydromantes* spp.) or Olm (*Proteus anguinus*) (BÖHME *et al.* 1999; CHIARI *et al.* 2012). For such species, habitat exposure risk is usually low and may only increase due to pesticide transport during specific weather conditions (DAVIDSON *et al.* 2002; DAVIDSON 2004) or leaching into subterranean habitats (BÖHME *et al.* 1999). For this EF, we awarded 2 'risk points' if habitat exposure risk was 'high', 1 if it was 'intermediate' and 0 if it is 'low'. In a first step, information was obtained from the literature including standard works on biology and ecology of European amphibians (GASC *et al.* 1997; BÖHME *et al.* 1999; TEMPLE & COX 2009) and from the IUCN Red List of Threatened Species (http://www.amphibians.org/redlist/). The literature-based estimates of habitat exposure are given in Appendix B. For detailed information for each species, see Appendix C.

Logistic regression analysis

For 13 species, the literature-based estimates were used for evaluating their habitat exposure risk. For the remaining 11 species, sufficient occurrence data were available to use logistic regression models to predict presence/absence as a function of land cover proportion type (LPA = CORINE land cover classes, which reflect areas where pesticides are regularly applied; see chapter 2.1). When the presence of a species positively correlated with LPA, a regular occurrence in agricultural landscapes was suggested. Hence, 2 'risk points' were awarded. 1 'risk point' was given if there was no significant trend, and 0 'risk point' if presence was negatively affected by the chosen land cover classes, so that it can be suggested that species usually do not occur within agricultural landscapes. For presence data, we collected all available coordinates of occurrence for Annex II amphibian species using the following databases: 'Global Biodiversity Information Facility', GBIF (http://data.gbif.org/), 'HerpNET' (http://www.herpnet.org/), and the 'Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity', RACE (http://www.bd-maps.eu). Occurrence data were corrected for duplicates and implausible records. For species with > 100 records (n = 5), we randomly chose a subset of 100 locations for each species. For species with less than 100 but more than 10 records (n = 6), we considered all locations (see Appendix D). 10 is the minimum sample size per predictor in logistic regressions (AGRESTI 2007). We set a 1 km buffer around each presence record to account for potential migration and dispersal. We are aware of the fact that distances of both home ranges and dispersal capacities can remarkably differ among amphibian species. However, according to JEHLE & SINSCH (2007) 1 km may be accepted as an average maximum range. Because of concern on spatial autocorrelation, presence records had to be at least 2 km apart to ensure that the 1 km circles do not overlap. Consequently, species with less than 10 suitable presence points (*i.e.*, whose 1 km buffers do not overlap) were not considered in further analysis (n = 13).

In a subsequent step, for each species that was considered in the analysis, the same number of absence as presence points was created. For doing so, we used a random sample of locations from SAC within the species' natural range (http://www.iucnredlist.org/amphibians), but where the considered species is not listed. Also absence points had to be at least 2 km apart and 1 km buffers were set. Finally, as predictor for the presence/absence of a species, the proportion of LPA was calculated within all buffers. Spatial data were processed using ArcMap 10 (Esri®).

'Evaluation factor' for migration behavior (EF 2)

Here, the question was how likely individuals of a species could pass agricultural land just when pesticides are applied? Although we are aware that pesticide use differs in agricultural areas, we surveyed the literature if species conduct relatively long annual migrations (from the hibernacula to the breeding sites, from breeding sites to the summer habitats, and back to the hibernacula) or have a more sedentary behavior. At a rough estimate, migratory species are active during most part of the vegetation period and therefore at higher risk of agrochemical contact than more sedentary species (see BERGER *et al.* 2012, 2013; BRÜHL *et al.* 2013). For example, the reproductive proportion of a *Triturus, Lissotriton, Rana,* or *Pelobates* population performs above mentioned annual migrations. Conversely, *Chioglossa lusitanica* only move short distances from the terrestrial habitats to the nearby reproduction streams (BÖHME *et al.* 1999).

For EF 2, we awarded 1-0 'risk points' each ('high', 'low') based on information from the literature including standard works on biology and ecology of European amphibians (Table 1; GASC *et al.* 1997; BÖHME *et al.* 1999; TEMPLE & COX 2009) and from the IUCN Red List of Threatened Species (http://www.amphibians.org/redlist/). For detailed information for each species, please see Appendix C.

'Evaluation factor' for breeding accumulations (EF 3)

Here, the question was if the reproductive proportion of a population accumulates at breeding sites when pesticide applications can occur? We reviewed the literature to see if species show breeding site accumulations. Hence, pesticide applications within this time may affect the main reproductive proportion of a population that is present for a defined time period in a small range (*i.e.*, the breeding site). For example, *Triturus, Lissotriton, Rana,* or *Pelobates* accumulate in early to late spring at breeding sites (*e.g.*, BÖHME *et al.* 1999), a time where different pesticides are applied in many cultivations (*e.g.*, BERGER *et al.* 2012; BRÜHL *et al.* 2013). Conversely, *Chioglossa lusitanica* or *Hydromantes* spp. have no breeding accumulations (BÖHME *et al.* 1999; CHIARI *et al.* 2012).

For EF 3, we awarded again 1-0 'risk points' each ('high', 'low') based on information from the literature including standard works on biology and ecology of European amphibians (Table 1; GASC *et al.* 1997; BÖHME *et al.* 1999; TEMPLE & COX 2009) and from the IUCN Red List of Threatened Species (http://www.amphibians.org/redlist/). For detailed information for each species, please see Appendix C.

Calculation of the 'pesticide risk factor'

Based on the three EF (EF 1 = 'habitat exposure risk'; EF 2 = 'temporal co-occurrence of migration' and EF 3 = 'breeding accumulations') each species could hypothetically receive 4 'risk points' (see Appendix B). EF 1 was partly literature-based, partly based on statistical analysis on presence/absence data and was suggested to be most important for potential exposure to pesticides. Hence, a species could score at maximum 2 'risk points' based on EF 1 but only 1 based on the remaining two EF. The 'risk points' defined the 'habitat exposure index' (HEI) for each species and based on the HEI and the proportion of LPA within the species' SAC, we finally calculated a 'pesticide risk factor' (PRF) for the habitat of each species using a modified formula from Rödder *et al.* (2009) under which a species habitat can score PRF 0-1:

PRF = HEI*area (%) / 400

National variations

To demonstrate national variations in pesticide exposure risk, which would argue for site-specific evaluations to avoid regional loss of amphibian biodiversity, we additionally calculated LPA within national SAC for all species, which occur in more than one EU member state. We tested if average proportions of LPA in national SAC and thereby exposure risk significantly differs between member states. Therefore, the proportions of LPA within national SAC of a species were compared using one-way ANOVA, followed by Bonferroni-corrected post-hoc tests (some data had to be Box-Cox-transformed prior calculations). All statistical analyses were performed with *R* and the package MASS (R DEVELOPMENT CORE TEAM, Vienna).

Results

LPA within the SAC and 'pesticide risk factors'

The proportion of LPA within the SAC ranges from less than 1 % in the Golden salamander (*Salamandra atra aurorae*) and the Corsican painted frog (*Discoglossus montalentii*) to more than 40 % in *Pelobates fuscus insubricus,* a subspecies of the Common spadefoot in Italy (Table 1; Fig. 1).

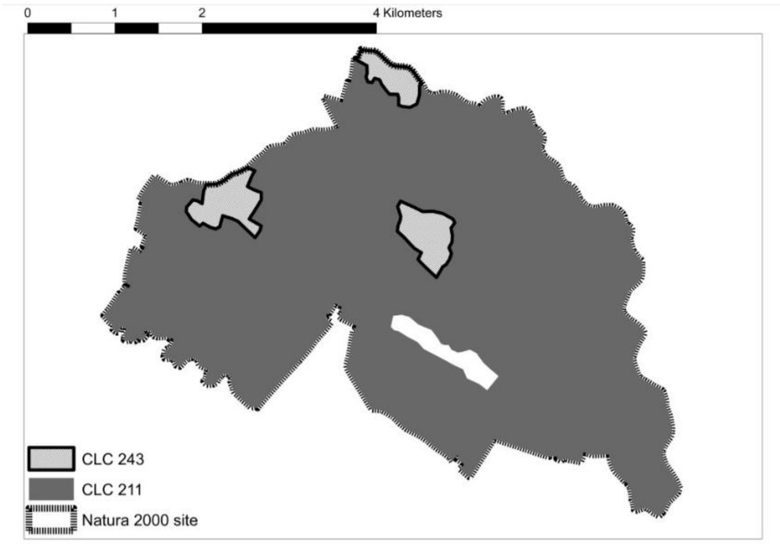

Fig. 1: Example of high agricultural land use within SAC.

The Natura 2000 site "IT 1110035: Stagni di Poirino – Favari", which has been created for the Common spadefoot subspecies *Pelobates fuscus insubricus*, is almost completely covered by CORINE land cover classes (CLC) 211 ("non-irrigated arable land") and 243 ("land principally occupied by agriculture with significant areas of natural vegetation").

Sufficient data for logistic regression analysis to confirm regular presence/absence of a species within cultivated landscapes could be obtained for 11 species. *Chioglossa lusitanica, Triturus cristatus, Hydromantes genei, H. imperialis,* and *Alytes muletensis* did not show significant trends. The presence of *Bombina bombina* and *Discoglossus galaganoi/D. jeanneae* was positively affected while the presence of *Lissotriton montandoni, H. strionatii, B. variegata and D. sardus* was negatively affected by the proportion of agricultural land use (Appendix B). The Common spadefoot *Pelobates fuscus insubricus* revealed the highest PRF (0.40), while nine species were supposed to be at low exposure risks to pesticide exposure (PRF = 0; see Table 1). The results show that species with more LPA within their SAC have also been assigned the high HEI values (cf. Appendices C and D). HEI values were strongly correlated with the proportion of LPA within the SAC (Spearman's rank correlation: S = 651.36, rho = 0.72, p < 0.001).

Priority species and global conservation status

Of the four European priority species, three are at low risk of pesticide exposure within their SAC except for *Pelobates fuscus insubricus* with over 40% agricultural land use within its SAC and the highest PRF. With regard to threat of species within their entire territories (but, for instance, *Triturus cristatus* has wider distributions outside the EU) on the basis of the IUCN Red List of Threatened Species, out of 24 Annex II amphibian species and subspecies, eight are endangered (according to the IUCN categories Critically Endangered, Endangered and Vulnerable), five are Near Threatened, while the remaining 11 are listed as of Least Concern (although in several of these the population trends are suggested to be decreasing) (Table 1). Out of the eight amphibian species with above-average PRF, six are listed as Least Concern. Only the newt *Triturus dobrogicus* is considered Near Threatened and the Italian agile frog (*Rana latastei*) even Vulnerable. Conversely, most of the species with lower risk of pesticide exposure are at higher risk of extinction as defined by the IUCN (11 out of 16; Table 1).

Variations at the national scale

For five species, significant differences in LPA proportions within national SAC could be revealed (Appendices D and E). Italian SAC that were created for *Discoglossus sardus* have significant greater proportions of LPA than French SAC (Appendix D). Bulgarian, Czech and Slovenian SAC that were created for *Bombina variegata* have significant greater proportions of LPA than SAC of several other EU member states (Fig. 2 A), likewise Czech and Estonian SAC that were created for *Triturus cristatus* (Fig. 2 B). For SAC that were created for *B. bombina*, again Bulgaria and the Czech Republic revealed highest LPA proportions (Fig. 2 C). Finally, Austrian and Bulgarian SAC that were created for *Triturus dobrogicus* have significant greater proportions of LPA than Hungarian and Slovakian SAC (Fig. 2 D).

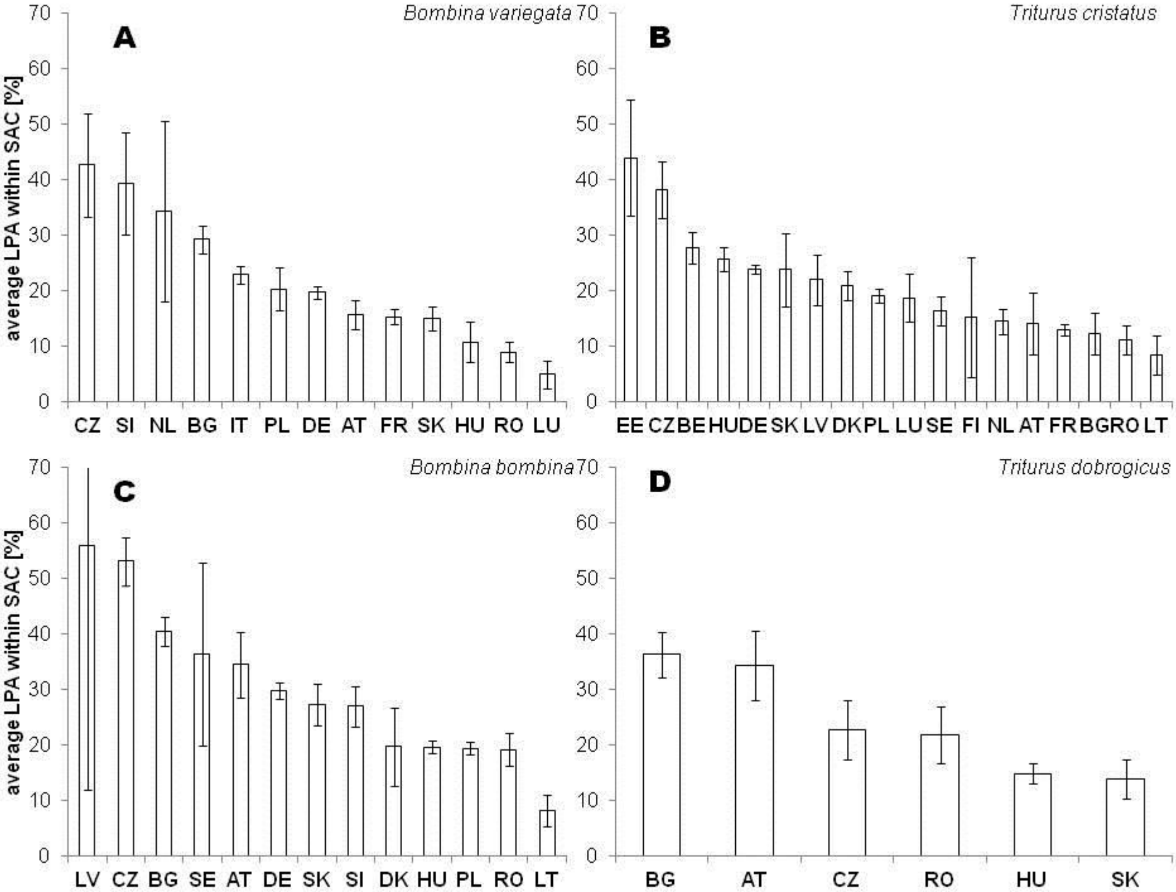

Fig. 2: National variations of average proportion of 'land use with regular pesticide applications' (LPA) (± SE) within national SAC that were created for Yellow-bellied toads (*Bombina variegata*) (A), Northern crested newts (*Triturus cristatus*) (B), Fire-bellied toads (*Bombina bombina*) (C) and Danube crested newts (*Triturus dobrogicus*) (D).

Note that SAC in Greece and the UK have not been evaluated due to lack of actual land cover data.

AT = Austria; BE = Belgium; BG = Bulgaria; CZ = Czech Republic; DE = Germany; DK = Denmark; EE = Estonia; FI = Finland; FR = France; HU = Hungary; IT = Italy; LU =Luxembourg; LT = Lithuania; LV = Latvia; NL = Netherlands; PL = Poland; RO = Romania; SE = Sweden; SI = Slovenia; SK = Slovakia

Discussion

Presence of species within cultivated landscapes

Our results suggest different occurrence probabilities for species within agricultural landscapes. This causes different risks for pesticide exposure, but one may also postulate that some species are more resistant to habitat alterations for agriculture (*e.g.*, mechanical and chemical cultivation techniques). However, in the past, populations of many amphibian species declined in Western Europe (HOULAHAN *et al.* 2000). Habitat destruction for and intensification of agriculture were and are main reasons for these declines, at least for lowland amphibians (MANN *et al.* 2009). Hence, recent absence of some species in agricultural landscapes may only be a result of earlier declines.

HEI values were highly correlated with LPA. A potential explanation therefore could be that (especially lowland) amphibian species that inhabit the types of land, which is suited for agriculture (*i.e.*, flat and mesic) (EF 1), are more likely to have life histories that involve long migrations to breeding sites (EF 2) where they congregate (EF 3). For instance, already GALLANT *et al.* (2007) stated that agricultural expansion is likely the single most important human activity affecting lowland amphibian populations.

Only 5 of the 11 logistic regressions agreed with the values assigned for EF1 based on the literature review. In most cases (*Alytes muletensis, Hydromantes genei, H. imperialis, Lissotriton montandoni*), the explanation therefore could be that in the natural range – where pseudo absence points were created for calculations – of a species that primarily lives in remote areas, also the buffers around the absence points contain few agricultural land use. Therefore, correlations were not significant and we assigned such species 1 risk point for EF 1 based on the regressions, but 0 risk points based on literature. Nevertheless, the strong correlation between HEI and LPA shows that our results are robust to any uncertainty in assigning the HEI scores, *i.e.*, if only the calculated LPA within the SAC would be regarded for PRF calculations, the results would be very similar.

Conservation status vs. need for protective measures?

Our results show that nearly all species with higher risk of pesticide exposure are listed as Least Concerned under the IUCN Red List of Threatened Species. The global IUCN status is equivalent to the European Red List status by TEMPLE & COX (2009). Conversely, species that are threatened within their entire territories and in Europe as well as European priority species mainly are at lower exposure risk – however, with some exceptions. Apparently, their specific SAC provide sufficient protection regarding pesticide use (but note that the situation outside the SAC has not been evaluated). This may primarily be explained by the fact that many of the threatened species occur in remote areas (see GASC *et al.* 1997).

Although amphibians with higher exposure risk are mainly not globally and Europe wide threatened, we identified at least two exceptions (*Triturus dobrogicus*, *Rana latastei;* Table 1). In addition, (i) the Painted frog *Discoglossus jeanneae*, which is seen as a subspecies of not threatened *D. galganoi* in the Habitats Directive, is listed as Near Threatened by the

IUCN. (ii) The subspecies of the Common spadefoot, *Pelobates fuscus insubricus*, which is at the highest risk of pesticide exposure and only inhabits a limited range in northern Italy (but see CROTTINI *et al.* 2007), has not yet been assessed by the IUCN, although it is extremely rare and at greater risk of decline (GASC *et al.* 1997). (iii) In front of the declaration of the Habitats Directive, four amphibian 'Key Species in the Council of Europe Area' were named for habitat conservation priorities, which are today all listed in Annex II of the Habitats Directive (*Bombina bombina, Alytes muletensis, Pelobates fuscus insubricus, Rana latastei:* CORBETT 1989). According to our results, three of them are at above-average risk of pesticide exposure; *P. f. insubricus* and *R. latastei* are even ranking first and second (Table 1). Especially the Italian subspecies of the Common spadefoot is at high risk of pesticide exposure within their SAC. This is related to the fact that in many countries where Common spadefoot toads previously lived in primary habitats like floodplains, they are forced today to accept secondary habitats like agricultural land (KIRSCHEY & WAGNER 2013).

Variations at the national scale

The significant differences between proportions of LPA within national SAC strongly argue for site and species-specifically evaluation to avoid national or regional loss of amphibian biodiversity. Especially in Czech and Bulgarian SAC that were created for Annex II amphibians, high proportions of LPA could be revealed. Site-specific detailed evaluations of pesticide exposure could start in these EU member states. This should include detailed information on species occurrence, cultivation and pesticide application practices.

Limitations of the risk evaluation and wich data are missing

For our risk evaluation at the European level, a lot of detailed data on pesticide use and species occurrence (especially within cultivated landscapes) are lacking. We must be content with CORINE land cover classes, literature and available occurrence data from the databases 'GBIF', 'HerpNET' and 'RACE'. With regard to land cover classes, the historical and actual detailed cultivation and thereby the detailed use of agrochemicals is unknown. For example, 5.4 % of the EU agricultural area is under organic cultivation (http://ec.europa.eu/agriculture/organic/eu-policy/data-statistics/index_de.htm), but some authorized substances for organic cultivation such as copper sulfate can also affect amphibian larvae (GARCÍA-MUÑOZ *et al.* 2009). Furthermore, the calculations of the present study can be seen as a “best-case scenario” because only the proportions of agricultural land have been considered, but not, for instance, the possibility of pesticides being transported over large distances away from the application areas during specific weather conditions (DAVIDSON *et al.* 2002; DAVIDSON 2004). In addition, on intensively used grass lands pesticides are regularly applied, but we did not consider CORINE land cover class 231 (“pastures”) Apart from this, quickly performed land use changes in Europe, especially by the increasing demand for agricultural land to grow ‘energy crops’, which includes ploughing up of grassland and cultivation in previously uncultivated land (FARGIONE *et al.* 2010; DAUBER *et al.* 2013) are not covered by the newest version of the CORINE data. Furthermore, there are lacking data with regard to species occurrence. On the one hand, records are mainly not from cultivated areas (*e.g.*, one can postulate that honorary conservationists and scientists report more species from protected and study areas outside the agricultural landscape). On the other hand, for some species almost no detailed occurrence data are currently available (*e.g.*, for *Rana latastei* only four records in ‘GBIF’, ‘HerpNET’ and ‘RACE’). Taken together, data on (1) historical and actual detailed land use, (2) detailed agrochemical use and residues and (3) species occurrence are necessary to conduct a risk assessment. This data could be obtained if monitoring programs would be realized.

Conclusions

It might be pragmatic to establish monitoring programs with regard to the potential exposure of habitats with pesticides (and occurrence of populations within agricultural used areas) in Habitats Directive management plans for – at least – SAC that were created for the following species, which are already globally threatened: Italian agile frog (*Rana latastei*), the Common spadefoot subspecies *Pelobates fuscus insubricus,* Danube crested newt (*Triturus dobrogicus*), and Painted frogs allocable to *Discoglossus jeanneae*. Such monitoring actions may include simple mapping of species and land use changes, but also regular laboratory analysis of water, sediments, and soil from their habitats. The guideline 4333 from the ‘Association of German Engineers (VDI)’ (see BÖLL *et al.* 2013) provides standard monitoring instructions to assess potential effects (mainly changes in pesticide use) from the cultivation of genetically engineered plants on amphibian populations. This guideline could serve as a model for monitoring effects of general use of agrochemicals on amphibian populations.

The above mentioned monitoring actions may be also necessary in SAC, which were created for amphibian species that are not globally threatened because populations of Annex II amphibians, which are still common in cultivated landscapes, may decline if effects of agrochemical use within their SAC will not be taken into account. Because most Annex II amphibians have their main distribution within the EU, potential declines due to agrochemical use may qualify for higher IUCN categories in the near future. Furthermore, although we did not consider many other "common" European amphibian species in the present study, recent studies highlight the potential of Annex II amphibian species as "umbrella species" (DENOËL *et al.* 2013), so that "common" amphibians species can benefit from monitoring and subsequent conservation actions within SAC.

Observed variations at national scale argue for site-specific evaluations of SAC to avoid regional loss of amphibian biodiversity. Apart from legal constraints (many amphibian species are strictly protected under national and European law as they are listed in Annex IV of the Habitats Directive and in many European countries, all amphibians are protected by national laws), the precautionary principle requires such actions because global populations of most European amphibians are decreasing (see http://www.amphibians.org/redlist/).

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Acute toxic effects of the herbicide formulation used in cycloxydim-tolerant maize cultivation on embryos and larvae of the African clawed frog, *Xenopus laevis*

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Abstract

Most genetically engineered herbicide-tolerant crops are still awaiting approval in Europe. There is, however, a recent trend for the cultivation of cycloxydim-tolerant maize hybrids for use in maize production. We studied the acute toxic effects of the complementary herbicide Focus® Ultra on embryos and early-stage larvae of the African clawed frog (*Xenopus laevis*), an established anuran laboratory species for toxicity testing. The results indicate that the herbicide formulation is highly toxic for early larvae. Based on calculated teratogenic indices, the formulation seems to be non-teratogenic and also the minimum concentration inhibiting growth in embryos and larvae was close to the LC50 values. The data suggest that some standard aquatic test organisms like the rainbow trout are not in all cases appropriate to assess the risk for aquatic development of anurans. This is demonstrated by 96-h LC50 values, which are for rainbow trout more than 20-fold higher than for *X. laevis* larvae. Added substances should mainly be responsible for adverse effects and not the a.i., so that pesticide risk assessment should always be based on the whole formulation. However, based on worst-case predicted environmental concentrations for surface waters, there is apparently a large safety margin in field use of Focus® Ultra if buffer strips are regarded, but calculated concentrations are only based on the a.i. and not on the more toxic added substances.

Keywords: Focus® Ultra, FETAX, amphibian decline, pesticides, corn

Introduction

Amphibian populations are declining globally at alarming rates (STUART *et al.* 2008). Different factors, often operating in tandem, such as habitat destruction, invasive species but also environmental contamination are often cited to play a role (COLLINS & STORFER 2003). In particular, pesticides reach the habitats of amphibians by various ways. These substances are known to cause toxic effects on all stages of amphibian development in both aquatic and terrestrial habitats (MANN *et al.* 2009). In Europe, maize cultivation has increased markedly in the last few decades, especially for fodder crop production. For example, in Germany the cultivation area for maize increased > 20% in relatively short time, from 21,111 km² in 2009 to 25,642 km² in 2012 (FEDERAL BUREAU OF STATISTICS: https://www.destatis.de). Today, land use changes for biofuel production are among the main concern for conservationists, as this type of agriculture is expanding in fallow wasteland or former mining landscapes (DAUBER *et al.* 2013). These lands are, among others, necessary secondary habitats and prerequisites for amphibian survival after the tremendous anthropogeneous changes of the landscape. In the USA and many other countries, maize cultivation is dominated by genetically modified (GM) crops and their specific herbicides. In Europe, GM cultivation is marginal, because such crops are still undergoing approval (BÖLL *et al.* 2013). Instead, since the beginning of the millennium, cycloxydim-resistant maize hybrids (CTM) have gained growing importance (VANCETOVIC *et al.* 2009) enabling repeated applications of the cycloxydim-based pesticide Focus® Ultra (BASF), which is also used by foliar spraying against perennial grasses in rape, sugar beet, potato, green bean, and field bean cultivations (EFSA 2012). For example, in 2012 the domestic sales for cycloxydim in Germany have been 10-25 tons of active ingredient (a.i.) and the export has been 100-250 tons of a.i. (BVL 2013). The effects of this herbicide formulation on amphibians remained unstudied. We conducted the first experimental trials to investigate the toxicity of Focus® Ultra on embryos and early larvae of the African clawed frog (*Xenopus laevis*). The developmental stages of *X. laevis* can be used as surrogates for other aquatic organisms because of their high sensitivity to pesticides and their availability as a laboratory species (ASTM 1998; BANTLE *et al.* 1998, 1999; WAGNER *et al.* 2013).

Material and methods

African clawed frog (*Xenopus laevis*) is a pipid anuran amphibian from southern Africa. The larvae are obligate suspension feeders pumping high amounts of water through their buccopharynx with the suggested consequence of tremendously increased contact to the compounds (VIERTEL 1990, 1992). Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of *X. laevis*.

Focus® Ultra is a selective herbicide containing 100 g/L (=10.8%) of cycloxydim (CAS 101205-02-1) as an active ingredient. Cycloxydim is a cyclohexene oxime herbicide, *i.e.*, it inhibits acetyl-CoA carboxylase in grasses while dicotyle plants and CTM are not affected (BURTON *et al.* 1989). Added vehicles to the formulation Focus® Ultra include 50% of Solvent Naphtha (a flammable liquid mixture of hydrocarbons, CAS 64742-95-6) and 2.4% of dioctyl sodium sulfosuccinate (CAS 577-11-7). For further information on the a.i. and both added substances, see the Pesticide Properties DataBase (PPDB) of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

All experiments were conducted in a climate chamber at 23 ± 1 °C and 12:12-h light-dark cycle. Embryo tests started with eggs at NF (NIEUWKOOP & FABER 1956) stages 8-11 and were terminated after 96-h when the embryos had reached NF stage 46. In accordance with YU *et al.* (2013), jelly coats of eggs were not removed because of concerns that the dejelling L-cysteine would induce teratogenic effects and to study a more natural development. Based on prior range-finding studies, concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 1.5 mg a.i./L were selected for embryo testing, which corresponds to 0, 1, 2.5, 5, 10, and 15 mg formulation (= Focus® Ultra) per litre. The embryo test procedure according to the FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) protocol (ASTM 1998) was applied, *i.e.*, four controls were used, test concentrations were duplicated, and the solutions were renewed every 24-h (static renewal).

The tests with larvae started at NF stage 47. The trials were terminated after 96-h when the larvae had reached NF stage 48. Only non-malformed larvae with normal behaviour were introduced. Based on prior range-finding studies, nominal concentrations of 0, 0.001, 0.01, 0.1, 0.25, and 0.5 mg a.i./L were applied, which corresponds to 0, 0.01, 0.1, 1, 2.5, and 5 mg formulation/L. Tests with early larvae were conducted using 5-L all glass aquaria, each containing one litre of test solution and 10 larvae. Test concentrations were triplicated and the solutions were renewed every 24-h (static renewal). Larvae were fed with 2.5 mg Sera Micron®/animal/day.

All test solutions were freshly prepared with FETAX solution (see ASTM 1998). Conductivity, dissolved oxygen, and pH ranged from 1482 to 1492 µS/cm, 5 to 8 mg/L, and

6.5 to 7.2, respectively. The average ammonia concentration was 0.15 mg/L with a range of 0.1 to 0.2 mg/L. For quality assurance, cycloxydim stock concentration of 100 mg a.i./L was measured using HPLC (Thermo Finnigan® TSP with UV2000 detector, P4000 pump, and AS3000 Auto sampler). 200 ml of solution were enriched on SPE columns (OASIS® HLB 6cc), conditioned with 3 mL of methanol and 3 mL of water, eluted with 4 mL of methanol, evaporated until dryness, and eventually absorbed into 100 µL of methanol:water (1:1). Sample injection was 20 µg/L. For higher nominal concentrations (*i.e.*, 1.5, 1.0, 0.5, 0.25, and 0.1 mg a.i./L), 15, 10, 5, 2.5, and 1 mL of stock solution were filled with FETAX solution in a Duran® graduated flask to obtain 1 L of test solution. For lower nominal concentrations (*i.e.*, 0.01 and 0.001 mg a.i./L), 10 and 1 mL of previously diluted 1 mg a.i./L test solution were filled with FETAX solution in a Duran® graduated flask to obtain 1 L of test solution.

Mortality, malformations (according to BANTLE *et al.* 1998) and growth inhibition were monitored after 96-h. Embryos and larvae were photographed after euthanization with 150 to 200 mg/L MS-222 (OECD 2009) and fixation in 5% formalin. The software "ImageJ" (NATIONAL INSTITUTE OF HEALTH) was used to measure head-tail-length (HTL). 96-h LC50 and 96-h TC50 values (median lethal and teratogenic concentration, respectively) were calculated with probit analyses. Significant differences were examined by overlap tests of 95% confidence intervals. Differences in mortality, malformations and HTL between groups were checked using one-way ANOVA (some data had to be Box-Cox transformed prior analysis to account for normal distribution and homogeneity of variances), followed by Bonferroni-corrected post-hoc-tests (for small sample sizes). For example, ASTM (1998) foresees a simple determination of significant differences by t-Test for grouped observations, but first conducting a one-way ANOVA for comparing mean values of different groups is statistically correct, as it accounts for the variance of the whole data set (DORMANN & KÜHN 2009). The software *R* and the package MASS were applied for statistical analyses (R DEVELOPMENTAL CORE TEAM, Vienna).

Results and discussion

Duplicate determination revealed only 86 and 89 % of a.i., respectively. These lower measured values are most probably based on the poor solubility of cycloxydim in water (53 mg/L in purified water at 20°C: EFSA 2012). Hence, diluted concentrations are expected to be lower than the calculated concentrations, but because only the stock solution has been measured, calculated concentrations were used to determine the endpoints. Survival of controls was always ≥ 90%. Total mortality of embryos started at 1.0 mg a.i./L (*i.e.*, 10 mg formulation/L) (ANOVA: F = 29.96, df = 1, P < 0.001). In the larvae experiment with the herbicide formulation, mortality was significantly increased at concentrations higher than 0.05 mg a.i./L (ANOVA: F = 70.79, df= 1, P< 0.001) (Fig. 1A+B). NOEC (No Observed Effect Concentration) for mortality in embryos was 0.5 mg a.i./L, in larvae 0.05 mg a.i./L (Fig. 1 A+B).

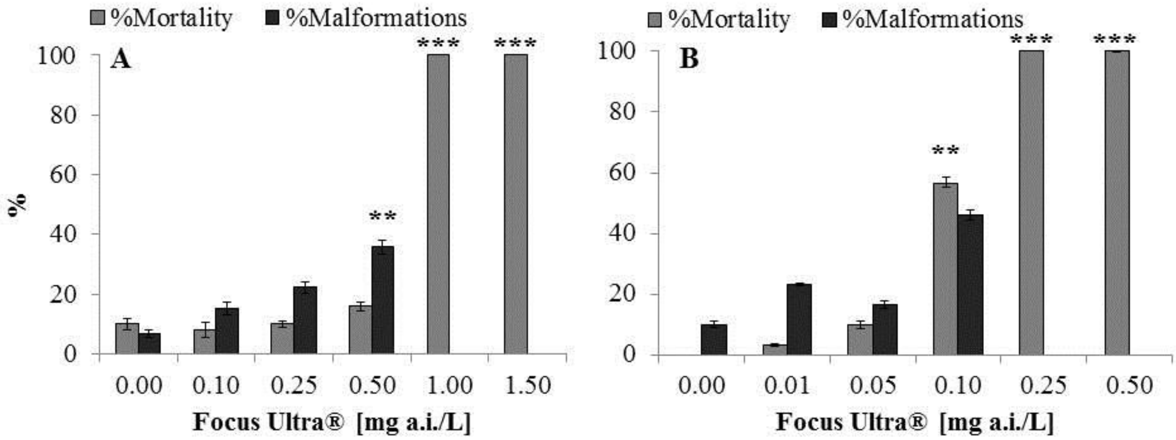

Fig. 1: Influence of Focus® Ultra on the 96-h mortality and malformation rates of *X. laevis* embryos (A) and early larvae (B)

Asterisks indicate significant differences to the control. All values are given ± standard error.

The 96-h LC50 values for the herbicide formulation suggest high toxicity of Focus® Ultra (0.1 mg/L < LC50 < 1 mg/L) for embryos and very high toxicity (LC50 < 0.1 mg/L) for early larvae if they were calculated for the amounts of a.i./L. If they 96-h LC50 values were calculated using the amounts of formulation/L, they suggest moderate toxicity of Focus® Ultra (1 mg/L < LC50 < 10 mg/L) for embryos and high toxicity (0.1 mg/L < LC50 < 1 mg/L) for early larvae (Table 1). Similar low LC50 values are rarely found in literature for *X. laevis* larvae, *e.g.*, 0.04-0.05 mg/L for the insecticide dieldrin (SCHUYTEMA *et al.* 1991). Overlap tests of 95% confidence intervals in the present study revealed that embryos were significantly more resistant than early larvae (Table 1). This finding is in accordance with EDGINTON *et al.* (2004) and probably caused by the absence of target organs in embryos such as functional external gills where the surfactants of pesticides can accumulate. It is suggested that the exposure of larvae is higher than in embryos due to the ventilation of the buccopharynx (see below).

Malformation rate in control embryos was < 7%, confirming validity of the present developmental toxicity test (ASTM 1998). Starting at 0.5 mg a.i./L (*i.e.*, 5 mg formulation/L), malformation rates in embryos significantly increased (ANOVA: $F = 38.06$, $df = 1$, $P < 0.001$) (Fig. 1A). This increase of morphological changes was exclusively observed at concentrations with increased mortality (LC50). Therefore, the calculated Teratogenic Index (TI = 96-h LC50/96-h TC50) was 0.94. According to BANTLE *et al.* (1999) the compound can be considered as non teratogenic. However, it has to be taken into account that mortality may camouflage teratogenic effects at an early stage before they are visible as diagnosable morphological changes. Therefore, the calculation of a TI may be generally misleading for some substances.

Tab. 1: Lethal and teratogenic concentration values of Focus® Ultra on embryos and early larval stages of *X. laevis*.
All values were calculated using probit analyses with 95% confidence limits stated.
NF stages – developmental stages of *X. laevis* at beginning of tests after NIEUWKOOP & FABER (1956)

Life stage	NF stages	Lethal concentration values		Slope of probit line
		96-h LC50 [mg a.i./L]	**96-h LC50 [mg formulation/L]**	
Embryos	8-11	*0.59* (0.51, 0.67)	*5.9* (5.1, 6.7)	5.38
		96-h TC50 [mg a.i./L]	**96-h TC50 [mg formulation/L]**	
		0.63 (0.41, 0.85)	*6.3* (4.1, 8.5)	3.73
		96-h LC50 [mg a.i./L]	**96-h LC50 [mg formulation/L]**	
Early larvae	46-48	*0.09* (0.07, 0.11)	*0.9* (0.7, 1.1)	47.98
		96-h TC50 [mg a.i./L]	**96-h TC50 [mg formulation/L]**	
		0.13 (0.04, 0.22)	*1.3* (0.4, 2.2)	-14.23

No significant dose-dependent increase in malformation rates in larvae has been observed. Malformation rate in controls was relatively high (3 out of 30 animals = 10%), but the highest malformation rate (6 out of 13 surviving larvae = 46.15%) occurred in the highest concentration within the surviving larvae (Fig. 1B). The TI was even lower than for embryos (0.69). The formulation can be considered to cause no developmental effects in larvae, however, with the restriction mentioned above for embryo toxicity. NOEC for teratogenicity in embryos was 0.25 mg a.i./L, in larvae (over) 0.10 mg a.i./L (Fig. 1 A+B).

Focus® Ultra significantly reduced growth in embryos at 0.5 mg a.i./L (*i.e.*, 5 mg formulation/L) and higher (ANOVA: $F = 38.1$, $df = 1$, $P < 0.001$) (Fig. 2A). Growth inhibition in larvae started at 0.1 mg a.i./L (*i.e.*, 1 mg formulation/L) (ANOVA: $F = 21.0$, $df = 1$, $P < 0.001$) (Fig. 2B). The minimum concentration inhibiting growth (MCIG) in embryos and larvae was close to the LC50 values. NOEC for growth retardation in embryos was 0.25 mg a.i./L, in larvae 0.05 mg a.i./L (Fig. 2 A+B).

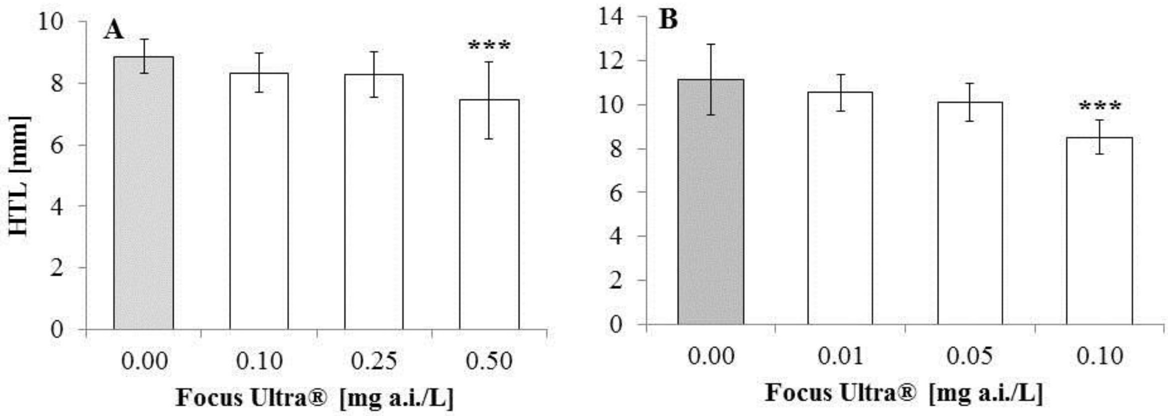

Fig. 2: Influence of Focus® Ultra on the 96-h growth of *X. laevis* embryos (A) and early larvae (B).

Asterisks indicate significant differences to the control. HTL = Head-tail-length. All values are given ± standard error.

The 96-h LC50 for the a.i. cycloxydim on the rainbow trout (*Oncorhynchus mykiss*), a teleost fish, is 220 mg/L (European Chemical Agency http://echa.europa.eu/documents/10162/cd799762-dd1a-4bb0-a772-bcd2d34da0b4) and the Safety Data Sheet of Focus® Ultra (BASF) states a 96-h LC50 value of 20.4 mg formulation/L for *O. mykiss*. The latter can be compared with the *X. laevis* results in mg formulation/L from the present experiments. Due to the low 96-h LC50 values for Solvent Naphtha for *O. mykiss* (1.03 mg/L: USEPA 2011 http://www.epa.gov/chemrtk/hpvis/hazchar/Category_Gasoline%20Blending%20Streams_December_2011.pdf) and dioctyl sodium sulfosuccinate (lowest 120-h LC50 of 0.4 mg/L for *O. mykiss*; GOODRICH *et al.* 1991), these compounds are understood to induce adverse effects. The most interesting finding in the present study is that the 96-h LC50 values for *O. mykiss* are more than 20-fold higher than for early *X. laevis* larvae.

It is suggested, that the different morphology of anuran larvae if compared with teleosts is responsible for this result. The body surface of teleosts is completely covered by scales or other dermal bones functioning as a barrier to the environment. The gills play a unique role in gas and ion exchange (FEDER & BURGGREN 1985; FENWICK 1989; EVANS *et al.* 1999, 2006). It does not wonder that toxic compounds are transported mainly via the fish gills (EVANS 1987; ERICKSON & MCKIM 1990). In contrary, the body surface of anuran larvae including their large tail fins play a role in gas exchange (BOUTILIER *et al.* 1992; ULTSCH *et al.* 1999). Additionally high amounts of water are in close contact with the epithelia of the oral cavity, the filter apparatus and the gills during ventilation (GRADWELL 1972a, b, c, 1975; WASSERSUG & HOFF, 1979, 1982; VIERTEL 1990, 1992). It has to be suggested that anuran larvae are much more exposed to their aquatic environment and as consequence to compounds than teleosts. So the toxic effects described are the result of both the compound and the specific properties of *Xenopus* larvae.

The data suggest that some teleosts species like *O. mykiss* are not sufficient surrogate organisms to assess the risk of pesticides such as Focus® Ultra to anuran larvae. A 10-fold safety factor is appropriate only for anuran embryos, but not for the early larvae. Here, a 100-fold safety factor is necessary. Due to the specific properties of anuran larvae, surrogate species are perhaps not sufficient for proper risk assessment of all pesticides and at least one anuran model organisms should be used. However, for most of the active ingredients such as organophosphates or carbamates (ALDRICH 2009) and some formulations such as glyphosate-based herbicides (WAGNER *et al.* 2013) standard aquatic test organisms seem to be sufficient for pesticide risk assessment, at least if a 10-fold safety factor is applied.

The EFSA (2012) states a worst-case foliar application scenario of Focus® Ultra of two times 400 g a.i./ha for sugar beets and beans (= dicot plants), which should be comparable with its use in CTM cultivations. The (simple) step 1 of the official surface water models of FOCUS (FOrum for the Coordination of pesticide fate models and their USe) states a worst-case predicted environmental concentration of cycloxydim for surface waters (PECsw; global maximum) of 0.26 mg a.i./L after two applications of 400 g a.i./ha (EFSA 2012). However, in the more realistic FOCUS step 2 and 3 scenarios, the worst case PECsw is reduced to 0.007 mg a.i./L after one application of 600 g a.i./L (unfortunately, no more precise models for two applications are available) in the northern European Union (EU), 0.009 mg a.i./L for the same application in the southern EU (step 2), and similarly 0.003 mg a.i./L for shallow bodies of water (ditches) (step 3) (EFSA 2012). Hence, there is apparently a large safety margin in field use for the herbicide, but more field data on real contamination levels and effects on autochthonous amphibians are necessary. Furthermore, all those calculated values are based on the a.i. (cycloxydim), but the formulation that is applied in the field, *i.e.*, the more toxic added substances are not considered. With regard that the added substances should be responsible for adverse effects and not the a.i., pesticide risk assessment should not only be based on the a.i. but always on the formulation.

Although Focus® Ultra is already labelled to be harmful to aquatic organisms, instructions and safety information foresee no buffer strips. For example in Germany, depending on the federal state, 5-10 m buffer strips are recommended, but critical amphibian breeding habitats in cultivated landscapes like vernal pools are not protected by no-spray buffer zones, as it is also the case in other countries like the U.S. (BATTAGLIN *et al.* 2009), so that, in these areas, the step 1 worst-case PECsw could be possible.

All autochthonous anurans in Europe are of the non-pipid type. The morphology of their larval buccopharynx is quite different from *Xenopus*. They are also suspension feeders, however, exploiting a much broader range of food sources than *Xenopus*. Some of them are bottom feeders ingesting sediment and detritus, some are facultative macro feeders. Experimental data demonstrate that they pump less water through their buccopharynx than *X. laevis* (VIERTEL 1990, 1992). The influence of these preconditions on toxic action of Focus® Ultra will be investigated separately.

In conclusion, (1) studies with rainbow trout are not appropriate to assess the risk of Focus® Ultra to anuran development. (2) Added substances should be mainly responsible for adverse effects and not the a.i. (cycloxydim). (3) Although the present data from laboratory experiments demonstrate high toxicity of Focus® Ultra to *Xenopus* larvae, there is apparently a large safety margin in field use for the herbicide (based on the EEC). However, these EEC are only calculated for amounts of a.i./L, but the more toxic added substances are not considered. (4) Buffer strips are recommended to prevent direct over-spraying of small ponds, which could cause high local concentrations of the compound and result in catastrophic consequences for amphibian reproduction. (5) Most anurans are not from the family Pipidae and have other larvae types, so that the effects of Focus® Ultra on aquatic life stages of non-pipids have to be investigated.

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Acute toxic effects of the herbicide formulation Focus® Ultra on embryos and larvae of the Moroccan painted frog, *Discoglossus scovazzi*

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Abstract

Intensification of agriculture is a serious threat to biodiversity. Especially pesticide use is increasing and numerous studies have shown deleterious effects on amphibians. We investigated acute toxic effects of the cycloxydim-based herbicide formulation Focus® Ultra on embryos and early larvae of the Moroccan painted frog (*Discoglossus scovazzi*). Clinical signs occurred at 4 and 8 mg a.i. /L in embryos and at 1, 1.5 and 2 mg a.i./L in larvae, which may result in elevated mortality in the field. Growth inhibition starting at 2 mg a.i./L in the embryos and at 0.25 mg a.i./L in the larvae was understood as sign of toxicity (retardation) and not as sign of teratogenicity. Connection to teratogenesis remained unclear though body size reduction occurred at concentrations lower than 20% of the 96-h LC50 and at a Minimum Concentration to Inhibit Growth (MCIG) of only 17% of the 96-h LC50. Starting at 2 mg a.i./L mortality in the embryos significantly increased and at 1.5 mg a.i./L in the early larvae. Mortality of larvae was enhanced during the first 24 h of exposure to 1.5 and 2 mg a.i./L. Morphology of the embryos remained unobtrusive. In contrary malformations significantly increased in the early larvae starting at 1 mg a.i./L, a concentration free of lethal effects. Larvae were significantly more sensitive than embryos, probably because exposure is higher due to the ventilation of the buccopharynx. Focus® Ultra induced comparable lethal and immobilization effects in *D. scovazzi* as it does to standard test organisms in pesticide approval.

Keywords: cycloxydim, Amphibia, anuran larvae, herbicide-tolerant, pesticide

Introduction

Increasing pesticide use is well known to have adverse effects on biodiversity (GEIGER *et al.* 2010), especially in freshwater ecosystems (BEKETOV *et al.* 2013). For example, pesticide residues can be found in most European surface waters above environmental quality standards (MALAJ *et al.* 2014) and the "National Action Plan on Sustainable Use of Plant Protection Products" of the German Federal Ministry of Food, Agriculture and Consumer Protection states the same, especially for small water bodies, which are situated in the agrarian landscape (BMEL 2013). In such small ponds, pollutants can accumulate and they are critical breeding habitat for most amphibian species (MANN *et al.* 2003; BÖLL *et al.* 2013). Worldwide, amphibian populations are decreasing at alarming rates (HOULAHAN *et al.* 2000; STUART *et al.* 2008). Although different other factors, such as habitat destruction, invasive species and emerging infectious diseases – including their interactions – are probably at work (COLLINS & STORFER 2003), environmental contamination, especially the increasing use of pesticides, is discussed to be one reason for the observed declines (COLLINS & STORFER 2003; BOONE *et al*. 2007). At environmentally relevant concentrations, pesticides are known to cause adverse effects on all stages of amphibian development in both aquatic and terrestrial habitats (reviewed in MANN *et al.* 2009) although their contribution to population or species decline remains unclear due to the current lack of data and uncertainties in identifying causative agents of decline (SCHMIDT 2004; WAGNER *et al*. 2013). Terrestrial life-stages can be directly over-sprayed (BRÜHL *et al.* 2013), come in contact with contaminated soil or plant material (BRÜHL *et al.* 2011) or feed on over-sprayed invertebrates (MCCOMB *et al.* 2008). Pesticides can find their way into habitats of aquatic life-stages by different ways, for instance, direct over-spraying (THOMPSON *et al.* 2004), run-off (EDWARDS *et al.* 1980) and drainage (BROWN & VAN BEINUM 2009) or drift (DAVIDSON 2004). For the aforementioned reasons – habitat contamination with pesticide residues and demonstrated adverse effects on amphibians – the new European regulation for plant protection products (EC No. 1107/2009) requires the risk to amphibians to be assessed using "all available information", but to date, in pesticide regulation practice in Europe and the United States, risk for amphibians is assessed by the results gained with standard test organisms, *i.e.*, mammals and birds for terrestrial life-stages, fish and aquatic invertebrates for aquatic life-stages (RELYEA 2011). Although safety factors of 10 to 100 are considered, depending on the studied species and endpoints, in several cases they seem to be insufficient (WAGNER *et al*. 2013), which is not surprising with regard to the physiological and ecological differences between amphibians and standard test organisms (RELYEA 2011, WAGNER *et al*. 2014). Amphibians absorb xenobiotics several times faster over their highly permeable skin than, for instance, mammals (QUARANTA *et al.* 2009; BELLANTUONO *et al*. 2014). Likewise, the body surface of aquatic life-stages, especially anuran larvae, already plays a crucial role in gas exchange (BOUTILIER *et al.* 1992; ULTSCH *et al.* 1999) and high amounts of water are in close contact with the epithelia of the oral cavity, the filter apparatus and the gills during ventilation (GRADWELL 1972a, b, c, 1975; WASSERSUG & HOFF, 1979, 1982), with significantly different pumping rates depending on the species (VIERTEL 1990, 1992). Conversely, the body surface of (adult) teleost fish, which are usually used as surrogates for acute toxic effects, is completely covered by scales or other dermal bones functioning as a barrier to the environment and, also in fish larvae with a more permeable skin, the gills play a unique role in gas and ion exchange (FEDER & BURGGREN 1985; FENWICK 1989; EVANS *et al.* 1999, 2006) and also absorption of xenobiotics (EVANS 1987; ERICKSON & MCKIM 1990). Hence, aquatic life-stages of amphibians are more exposed to their aquatic environment and as consequence to compounds than most teleosts such as rainbow trout (*Oncorhynchus mykiss*), a common standard test organism.

Recent studies rightly highlight the need for a better understanding of pesticides on amphibians under natural conditions (EDGE *et al.* 2013, 2014) because different abiotic and biotic factors can decrease or increase effects in the wild (WAGNER *et al.* 2013). However, in a first step to achieve a better risk assessment for a pesticide, which effects on amphibians has not been tested yet, laboratory model organisms for autochthonous amphibian species are needed to gain first results on acute toxic effects of a substance and draw causal links. Especially, the buccopharynx in anuran larvae differs species-specifically. Early developmental stages of the African clawed frog (*Xenopus laevis* [DAUDIN, 1802], Pipidae) are commonly used in scientific studies as surrogates for other aquatic organisms including other amphibian larvae because of their high sensitivity to pesticides and their availability as a laboratory species (ASTM 1998; BANTLE *et al.* 1998, 1999; OECD 2009). *Xenopus* larvae are obligate suspension feeders pumping high amounts of water through their buccopharynx with the suggested consequence of tremendously increased contact to the compounds (VIERTEL 1990, 1992). However, most anuran species are non-pipids and their larvae are only facultative suspension feeders, pumping less water through their buccopharynx than *X. laevis* (VIERTEL 1990, 1992). Here, we use early developmental stages of an anuran non-pipid model organism to assess the risk of a commonly used herbicide formulation.

Material and methods

Less amphibian-toxicological studies have been conducted with herbicides, although they are used in higher amounts compared to, for instance, insecticides (WEIR *et al.* 2012). Therefore, we chose an herbicide formulation as test substance, which is commonly used in corn production. In the Americas and Europe, corn cultivation has already increased in the last few decades – especially for fodder crop production – but recently, corn cultivation is remarkably expanding for biofuel production (LANDIS *et al.* 2008). For example, in Germany the cultivation area for corn increased over 20% in relatively short time, from 21,111 km² in 2009 to 25,642 km² in 2012 (FEDERAL BUREAU OF STATISTICS: https://www.destatis.de).

In the Americas and other parts of the world, corn cultivation is mainly based on genetically modified crops with a resistance against glyphosate enabling repeated applications of glyphosate-based herbicides during cultivation (DUKE & POWLES 2008). Conversely, in most European countries, genetically modified crop cultivation is only marginally as most of such crops are still undergoing approval (BÖLL *et al.* 2013), with some exceptions such as Spain where nearly one-third of corn cultivation is with genetically modified plants (www.isaaa.org/). Since the beginning of this millennium, cycloxydim-resistant corn hybrids (which are not genetically modified) have gained growing importance (VANCETOVIC *et al.* 2009) enabling repeated applications of the cycloxydim-based pesticide Focus® Ultra (FO-UL), which is also used by foliar spraying against perennial grasses in conventional rape, sugar beet, potato, green bean, and field bean cultivations (EFSA 2012).

FO-UL is a selective herbicide containing 100 g/L (= 10.8%) of cycloxydim (CAS 101205-02-1) as active ingredient (a.i.). Added substances are 50% of Solvent Naphtha (CAS 64742-94-5) and 4% of sodium dioctylsulphosuccinate (CAS 577-11-7). Its a.i. cycloxydim is a cyclohexene oxime herbicide, *i.e.*, it inhibits acetyl-CoA carboxylase in grasses while dicotyle plants and cycloxdyim-resistant corn hybrids are unaffected (BURTON *et al.* 1989).

As an anuran model organism for non-pipid and facultative suspension feeding larvae, we used the Moroccan painted frog (*Discoglossus scovazzi* [CAMERANO, 1878], Alytidae) because of availability of eggs over the whole year which is not the case in Central European species. The spontaneous egg deposition and the relatively fast development of aquatic life-stages is a benefit for laboratory work. *D. scovazzi* is distributed in the Mediterranean region of Morocco and the Spanish exlaves Ceuta and Melilla. Before it has been assigned species rank using molecular data, it has been understood as a subspecies of *D. pictus* (OTTH, 1837), which occurs from northern Algeria to Tunesia, on Sicilia, Malta and was introduced in northern Spain and southern France (FROMHAGE *et al.* 2004). Pesticide exposure risk is high in the area of *D. scovazzi* as 68% of Morocco is agriculturally used (30,403,000 ha of 44,655,000 ha total area: http://faostat.fao.org), which is most of the non-desert area. So the present study may have a benefit for the species.

All experiments were conducted in a climate chamber at 23 ± 1 °C and a 12:12 h light:dark cycle. The test solutions were renewed every 24 h and the tests were terminated after 96-h. Water chemistry data (pH level, dissolved oxygen [%], nitrate and ammonium concentrations [mg/L]) were taken at beginning of each experiment and before renewal of test solutions. All tests solutions were freshly prepared with FETAX solution (see ASTM 1998). Egg clutches from *D. scovazzi* were provided by Prof. Dr. FRANK PASMANS (Gent University). The embryo experiments started with normally developed eggs at Gosner (GOSNER 1960) stages 8-9 (= blastula to early gastrula). The trials were conducted following the FETAX protocol (ASTM 1998) Petri dishes (60 mm in diameter) contained 10 mL of test solution and 25 embryos. Four controls were used and test concentrations were duplicated (ASTM 1998). The FETAX protocol was slightly modified. The jelly coats of the eggs were not removed because of concerns that L-cysteine could harm the embryos and to provide more natural conditions (YU *et al.* 2013). Furthermore, no positive control was used to prohibit cross contamination with 6-aminonicotinamide in the climate chamber. Calculated concentrations of FO-UL in the embryo tests – based on a dose range-finding study – were 0, 10, 15, 20, 40 and 80 mg formulation FO-UL/L containing 0, 1, 1.5, 2, 4 and 8 mg active ingredient/L. The embryos hatched in petri dishes (94 mm in diameter) containing 25 mL of FETAX solution and 25 embryos each. Experiments started with *D. scovazzi* individuals at Gosner stage 25 (= free swimming larvae) in accordance with the standard protocol of ASTM (2002). The tests were conducted using 5-L full glass aquaria, containing one litre of test solution and 10 larvae each. Only non-malformed larvae exhibiting normal behaviour were included. Control and test concentrations were triplicated. Larvae were fed with Sera Micron® (2.5 mg per animal/day) on a daily basis. Calculated concentrations of FO-UL in the larvae tests – based on a dose range-finding study – were 0, 2.5, 5, 10, 15 and 20 mg formulation FO-UL/L containing 0, 0.25, 0.5, 1, 1.5 and 2 mg active ingredient./L.

Embryos and larvae were euthanized with an overdose of MS-222 (150-200 mg/L according to OECD (2009), fixed in 5% formaldehyde, and photographed with a digital camera. Measurements of head-tail-lengths were conducted using the software “ImageJ” (NATIONAL

INSTITUTE OF HEALTH). Median lethal (LC50, mortality) and median teratogenic (TC50, malformations) concentrations values were determined with Probit analysis. Although 83-84% confidence intervals seem to be enough to give an approximate $\alpha = 0.05$ test (PAYTON *et al.* 2003), significant differences between LC50 values were examined by overlap tests of their 95% confidence intervals. Hence, our comparisons can be seen as conservative results. The goal of the Teratogenic Index (TI = LC50 values divided by the TC50 values) is toseparate the lethal action of a compound from his teratogenic potential. TI values < 1.5 should indicate low teratogenic potential of a substance because little or no separation exists between lethal and malformation inducing concentrations (according to BANTLE *et al.* 1999; FORT & ROGERS 2005). The results will demonstrate that TI is not representative in the present study. Significant differences in mortality, malformations and growth inhibition among treatment groups were examined using one-way ANOVA followed by Bonferroni-corrected posthoc tests. Some data sets had to be Box-Cox transformed prior to analysis to reach normal distribution and homogeneity of variances. The software *R* and the package “MASS” were applied for all calculations (R DEVELOPMENTAL CORE TEAM, Vienna).

For quality assurance, amounts of a.i. were measured in one dilution series using HPLC (Thermo Finnigan® TSP with UV2000 detector, P4000 pump, and AS3000 Auto sampler). 200 ml of solution were enriched on SPE columns (OASIS® HLB 6cc), conditioned with 3 mL of methanol and 3 mL of water, eluted with 4 mL of methanol, evaporated until dryness, and eventually absorbed into 100 µL of methanol:water (1:1). The sample injection concentration was 20 µg/L.

Results

The measured concentrations were slightly lower than the calculated. The difference was stable and did not reach the level of significance (79.9 ± 2.7 %). Therefore, the calculated concentrations are understood as representative taking into account that the exposure concentrations were lower. At the beginning of the tests, pH levels ranged from 6.5 to 6.8, dissolved oxygen from 93 to 100% and ammonium concentration was always 0 mg/L. At the end of the tests, pH levels were in the same range as measured at the beginning, dissolved oxygen ranged from 60 to 93%, nitrate concentrations from 0 to 10 mg/L and ammonium concentrations from 0.1 to 0.4 mg/L.

Mean survival of embryos in all controls was always ≥ 90% and 100% in larvae controls and malformation rates were ≤ 7% (no malformed larvae at all) confirming validity of the toxicity tests (ASTM 1998). Tapping the test animals at the tail with a blunt glass rod revealed abnormal behaviours such as twitching, convulsion and narcosis at 4 and 8 mg a.i./L in the embryos and at 1, 1.5 and 2 mg a.i./L in the larvae. These clinical signs could be observed over the whole study time, shortly after renewal of test substance through the next 24 h. For the larvae, the 24-h EC50 was calculated as 1.0 mg a.i./L (95% confidence intervals 0.9, 1.1 mg a.i./L). Starting at this concentration, clinical signs were significantly increased if compared to the control (ANOVA: $F_{1,13} = 46.86$, $P < 0.001$, Fig. 1). NOEC (No Observed Effect Concentration) for clinical signs were 2.0 mg a.i./L in embryos and 0.5 mg a.i./L in larvae (Fig. 1).

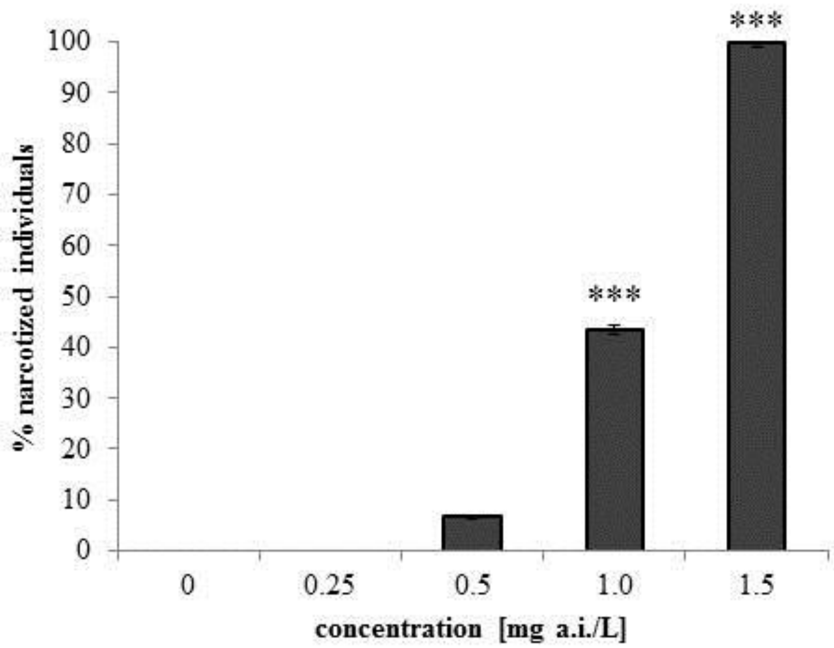

Fig. 1: Influence of Focus® Ultra on clinical signs of *D. scovazzi* early larvae 24 h after exposure.

Asterisks indicate significant differences to the control. All values are given ± standard error.

Based on mg a.i./L, FO-UL has been found to be "moderately toxic" for *D. scovazzi* embryos and early larvae (1 mg/L < LC50 < 10 mg/L: USEPA 2012) but only "slightly toxic" based on mg formulation/L (10 mg/L < LC50 < 100 mg/L: USEPA 2012) (Table 1). Overlap-test of the 95% confidence intervals revealed that early larvae were significantly more sensitive to FO-UL exposure than the embryos (Table 1). Starting at 2 mg a.i./L, mortality in the embryos significantly increased (ANOVA: $F_{1,12} = 50.56$, $P < 0.001$: Fig. 2A) and starting at 1.5 mg a.i./L in the early larvae (ANOVA: $F_{1,16} = 34.64$, $P < 0.001$: Fig. 2B). NOEC for mortality was 1.5 mg a.i./L in embryos and 1.0 mg a.i./L in larvae (Fig. 2). Rapid mortality of larvae was observed during the first 24 h of exposure to 1.5 and 2 mg a.i./L, the two highest concentrations. No significant increase in malformations was seen in embryos after exposure to FO-UL (ANOVA: $F_{1,10} = 1.35$, $P > 0.05$: Fig. 2A). In contrary, incidences significantly increased starting at 1 mg a.i./L in early larvae (ANOVA: $F_{1,13} = 42.50$, $P < 0.001$: Fig. 2B), a concentration free of lethal effects (Fig. 2B). In consequence the calculated Teratogenic Indices (TI = 96-h LC50/96-h TC50) were < 1.5. NOEC for teratogenicity was above 8.0 mg a.i./L in embryos and 0.5 mg a.i./L in larvae (Fig. 2).

The Minimum Concentration that Inhibits Growth (MCIG) of embryos was 2.0 mg a.i./L and was close to the LC50 value of 2.92 mg a.i./L (ANOVA: $F_{1,209} = 17.07$, $P < 0.001$, Table 1, Fig. 3A). In contrary, growth of the larvae was significantly decreased already at 0.25 mg a.i./L, *i.e.*, a concentration more than five times lower than the LC50 value of 1.45 mg a.i./L (ANOVA: $F_{1,122} = 81.10$, $P < 0.001$, Table 1, Fig. 3B). NOEC for growth retardation was 1.5 mg a.i./L in embryos and below 0.25 mg a.i./L in larvae (Fig. 3).

Tab. 1: Lethal and teratogenic concentrations and Teratogenic Indices (TI) of FO-UL on embryos and larvae of *D. scovazzi*. a.i. = active ingredient, formulation = FO-UL

All values are presented with 95% confidence intervals in parenthesis. TI values < 1.5 indicate low teratogenic potential of a substance.

Life-stage	**96-h LC50** (mg a.i./L)	**96-h LC50** (mg formulation/L)	**96-h TC50** (mg a.i./L)	**96-h TC50** (mg formulation/L)	**TI**
Embryos	**2.92** (2.43; 3.41)	**29.2** (24.3; 34.1)	**6.74** (3.21; 10.27)	**67.4** (32.1; 102.7)	**0.43**
Early larvae	**1.45** (1.33; 1.57)	**14.5** (13.3; 15.7)	**1.74** (1.21; 2.27)	**17.4** (12.1; 22.7)	**0.83**

Discussion

The difference between calculated and measured concentrations of the active ingredient was most probably due to the poor solubility of cycloxydim in water (53 mg/L in purified water at 20°C: EFSA 2012). According to the safety data sheet of FO-UL, also both added substances are either poor soluble (sodium dioctylsulphosuccinate: 15 mg/L at 25 ºC) or even insoluble (Solvent Naphtha) in water. The decreased oxygen and the increased ammonium concentrations after 24 h were most probably caused by the increased metabolism of the larvae producing faeces and by amounts of unconsumed food. Low mortality in embryo controls (9.0 ± 0.6 %) and larvae controls (0%) demonstrated the validity of the study.

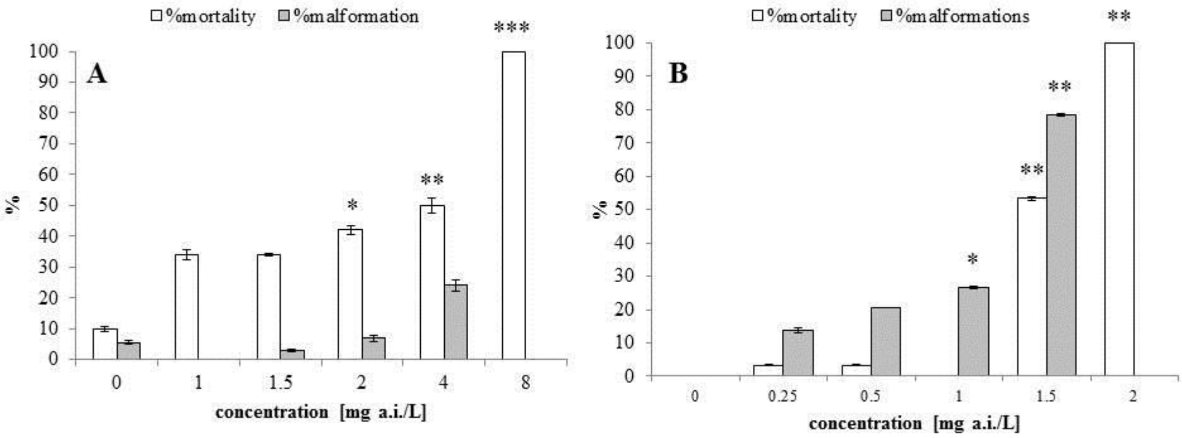

Fig. 2: Influence of Focus® Ultra on the 96-h mortality and malformation rates of *D. scovazzi* embryos (A) and early larvae (B)

Asterisks indicate significant differences to the control. All values are given ± standard error.

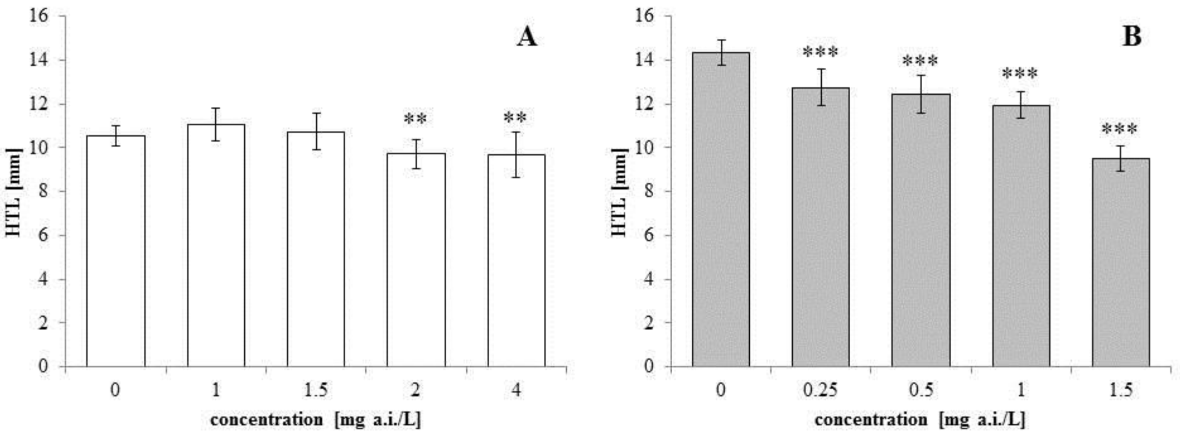

Fig. 3: Influence of Focus® Ultra on the 96-h growth of *D. scovazzi* embryos (A) and early larvae (B)

Asterisks indicate significant differences to the control. All values are given ± standard error.

Twitching, convulsion and narcosis were also observed by YU *et al.* (2013) during exposure of *X. laevis* embryos to α-cypermethrin and by MANN & BIDWELL (2001) during exposure of different anuran larvae (*X. laevis, Rhinella marina, Crinia insignifera, Heleioporus eyrei, Limnodynastes dorsalis* and *Litoria moorei*) to agricultural surfactants. BERNABÓ *et al.* (2011) observed irregular swimming only in malformed *Rana dalmatina* larvae after exposure to the insecticide chlorpyrifos. Conversely, this abnormal behaviour was also observed in *D. scovazzi* larvae in the present study without external malformations. An effect of an added substance was suggested for narcosis. Starting at a concentration of 1 mg a.i./L, clinical signs were significantly increased; however, larval mortality remained normal at this concentration (cf. Fig. 1 and Fig. 2B). In the wild, the observed clinical signs may result in an increased mortality in larvae as consequence of properties of the habitat. With regard to pesticide approval, immobilization in the standard test organism *Daphnia magna* is, however, comparable (safety data sheet: 48-h EC50 of 15.5 mg formulation/L = 1.55 mg a.i./L).

With regard to the malformation rates and mortality, calculated TI values for embryos and larvae were < 1.5. On this basis, FO-UL can be formally considered as non-teratogenic (BANTLE *et al.* 1999; FORT & ROGERS 2005). However, this approach is only valid when teratogenicity and mortality are based on different mechanisms. For compounds whose toxic profile and pathophysiological actions on biological systems are unknown, TI should be applied with cautiousness. It has to be taken into account that mortality may camouflage developmental effects which at lower doses are less severe allowing the embryos or larvae to survive and to exhibit morphological changes. It was not excluded that this was true for the embryos in the present study. All in all, the calculation of a TI is misleading for the interpretation of the data in the present study. A compound was considered teratogenic by BANTLE *et al.* (1999) when the MCIG was significantly different from controls at concentrations below the 30% level of the MAS 96-h LC50 (MAS = experiments with use of a Metabolic Activation System). The MCIG for early larvae of *D. scovazzi* was only 17.24% of the 96-h LC50 in the present study, hence, FO-UL could be considered teratogenic to the larvae in the present study with regard to growth inhibition on the basis of BANTLE *et al.* (1999). In parallel to mortality and teratogenicity, it is questionable if action on growth and teratogenicity are closely linked in all cases. Therefore, in the present study growth inhibition is understood as a sign of retardation and not automatically as a sign of teratogenicity.

Our data show that exposure of early developmental stages to the commonly used FO-UL formulation resulted in life-stage dependent and dose related effects. The finding that embryos are significantly more resistant than early larvae is in accordance to previous studies on the toxicity of herbicides to anuran larvae (*X. laevis, Lithobates clamitans, L. pipiens, L. sylvaticus, Anaxyrus americanus*: EDGINTON *et al.* 2004; HOWE *et al.* 2004). The authors explained their observations as consequence of the absence of target organs such as functional gills in embryos where especially surfactants can accumulate. We additionally suggest that the exposure of larvae is higher than in embryos due to the ventilation of the buccopharynx (VIERTEL 1990, 1992).

It has been shown for several other pesticides that mainly the added substances (surfactants) provoked mortality and not the active ingredient (PUGLIS & BOONE 2011; WAGNER *et al.* 2013). This is also true for FO-UL. The 96-h LC50 value of the a.i. cycloxydim for the rainbow trout (*Oncorhynchus mykiss*), an aquatic standard test organism, is ten times higher than for FO-UL (safety data sheet: 20.4 mg formulation/L; 220 mg cycloxydim/L see European Chemical Agency http://echa.europa.eu/documents/10162/cd799762-dd1a-4bb0-a772-bcd2d34da0b4). Comparison of this 96-h LC50 FO-UL value with *D. scovazzi* leads to the conclusion that the embryos are even more resistant (96-h LC50: 29.2 mg formulation/L; Table 1) and that a 10-fold safety factor is sufficient for the larvae (96-h LC50: 14.5 mg formulation/L; Table 1). Which one of the two substances added to FO-UL – Solvent Naphtha or sodium dioctylsulphosuccinate – is mainly responsible for the adverse effects of the formulation should be separately investigated.

Rapid lethal effects in anuran larvae within the first 24 h after application were also observed by RELYEA (2005) for a Roundup® formulation. Therefore, studies on acute toxicity of herbicide formulations should be flanked by pesticide monitoring programs in the environment to predict concentrations based on worst-case scenarios such as washing out due to heavy rain fall. Furthermore, even fast dissipation of pesticides by microbial degradation or adsorption to sediments may not be sufficient to protect aquatic life-stages of anurans against temporarily high concentrations inducing acute toxic effects as proposed by BERNAL *et al*. (2009).

Embryos of *X. laevis* are about five times (96-h LC50 of 0.59 mg a.i./L; 95% confidence intervals 0.5, 0.7 mg a.i./L) and early larvae about 15 times (96-h LC50 of 0.09 mg a.i./L; 95% confidence intervals 0.07, 0.11 mg a.i./L) more sensitive to FO-UL than those of *D. scovazzi* (N. WAGNER *et al*., unpublished data). Such species-specific responses to herbicide exposure in anuran larvae have been previously reported (WILLIAMS & SEMLITSCH 2010; FUENTES *et al*. 2013). This could argue for *X. laevis* to be a good sentinel species for other anurans because embryos and larvae were often significantly more sensitive than early developmental stages of other anurans (see MANN & BIDWELL 2000, 2001; EDGINTON *et al*. 2004). The morphology of the larval buccopharynx of *D. scovazzi* and of other non-pipid species is quite different from *Xenopus*. They are also suspension feeders, however, exploit a much broader range of food sources than *Xenopus*. Some of them are bottom feeders ingesting sediment and detritus, some are facultative macro feeders. Experimental data on ingestion and filtering rate demonstrate that non-pipid species pump less water through their buccopharynx than *X. laevis* (VIERTEL 1990, 1992). If the idea is accepted that the amount of water, which is in contact with the larva is in connection with the exposure to dissolved compounds, *X. laevis* larvae are remarkably more exposed than the non-pipid larvae.

The (simple) step 1 of the official surface water models of FOCUS (FOrum for the Coordination of pesticide fate models and their USe) states a worst-case predicted environmental concentration of cycloxydim for surface waters (PECsw; global maximum) of 0.26 mg a.i./L after two applications of 400 g a.i./ha (EFSA 2012). However, in the more realistic FOCUS step 2 and 3 scenarios, the worst case PECsw is reduced to 0.007 mg a.i./L after one application of 600 g a.i./L (unfortunately, no more precise models for two applications are available) in the northern European Union (EU), 0.009 mg a.i./L for the same application in the southern EU (step 2), and similarly 0.003 mg a.i./L for shallow bodies of water (ditches) (step 3) (EFSA 2012). Based on these calculations, there is apparently a large safety margin in field use for FO-UL. Yet more field data on realistic contamination levels in the field and effects on other amphibian species are necessary to validate this apparent safety.

Conclusions

FO-UL was toxic to embryonic stages and early larvae of *D. scovazzi*, but based on PECsw values, there is apparently a large safety margin in field use In all endpoints the larvae were more sensitive than the embryos. FO-UL provoked clinical signs starting in larvae at sublethal concentrations. Clinical signs may result in elevated mortality under conditions in the field. It is not excluded that mortality under laboratory conditions camouflaged teratogenic effects of the test item especially in the embryonic stages. Growth retardation was understood as a sign of toxicity. Connection to teratogenesis remained unclear though it occurred at concentrations lower than 20% of the 96-h LC50.

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No developmental effects but time-lag mortality of environmentally relevant Focus® Ultra concentrations on *Xenopus laevis* larvae.

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Abstract

Corn production has remarkably increased in the last decades. Modern agrarian practices include cultivation of herbicide-tolerant crops, i.e. several applications of the complementary herbicide over the growth phase are possible, while in conventional corn production, herbicide application is restricted to the beginning of cultivation. The enhanced application time space increases the risk of non-target organisms to come into contact with the herbicide residues. Popular complementary herbicides for maize hybrids are cycloxydim-based. There is growing awareness about detrimental effects of pesticide use on biodiversity, especially on aquatic wildlife. We therefore examined effects of the cycloxydim-based herbicide formulation Focus® Ultra at doses of 0.1 and 0.5 mg formulation/L (0.01 and 0.05 mg active ingredient/L, close to the calculated LC5 and LC10 values) on early, intermediate and pre-metamorphic larval stages (NF stages 47, 51 and 57) of the anuran model organism *Xenopus laevis*. In addition, larvae were repeatedly exposed, i.e. at all considered life stages. Intensity of narcosis 24 h after exposition was growing with the larval stage demonstrating increased sensitivity of the older larvae. 48 h after exposition narcosis was mild and compared in all stages. Most of the narcotized larvae recovered. Narcosis may result in increased mortality under conditions in the field such as rise of predation risk. Mortality occurred with a time lag. Early and late larval stages were more sensitive to lethal action than intermediate larvae and repeatedly exposed larvae. Metamorphic success did not differ between the developmental stages. The herbicide did not induce effects on body size and on time to metamorphosis. No increase of deformation rates was seen. In conclusion, Focus® Ultra seems to affect anuran larvae more acutely than chronically.

Keywords: pesticide, herbicide, cycloxydim, maize, corn, anuran larvae

Introduction

Worldwide, amphibian populations showing unnatural negative trends with one third of the ca. 7000 recognized species threatened with extinction (ALFORD & RICHARDS 1999; HOULAHAN *et al.* 2000; STUART *et al.* 2008). Different factors including their interactions are at work and environmental pollution is one of the discussed reasons (COLLINS & STORFER 2003). In general, pesticide use is strongly affecting farmland biodiversity (GEIGER *et al.* 2010) and it is suggested that this is also true for amphibians that are persisting in cultivated landscapes (MANN *et al.* 2009) and reproduce in agricultural ponds (KNUTSON *et al.* 2004). In contrast to this assumption, recent studies have shown that amphibian populations from agrarian landscapes are more resistant than those from more remote areas (COTHRAN *et al.* 2013, HUA *et al*. 2013). COTHRAN *et al.* (2013) and HUA *et al*. (2013) assume that this is an evolutionary response to regular pesticide use, which may reduce the risk of pesticides for amphibians. Furthermore, causal relationships between pesticide use and declines of amphibian populations are poorly understood (SCHMIDT 2004; WAGNER *et al*. 2013). Nevertheless, in several field studies negative effects at the individual level have been found, which include development of abnormal gonads (MCCOY *et al.* 2008), decreased body size and mass at metamorphosis (ATTADEMO *et al*. 2014; WAGNER *et al*. 2014), stress and alterations in enzyme activity (ATTADEMO *et al*. 2007, 2011, 2014; LAJMANOVICH *et al*. 2010, 2011), which could lead to increased infection risk with pathogens (ATTADEMO *et al*. 2011). In addition, numerous laboratory and mesocosm studies have already shown deleterious effects of pesticides at environmentally relevant concentrations on amphibians, mainly on anuran larvae (HOWE *et al*. 2004; RELYEA 2009; BERNABÒ *et al*. 2013). Beside acute toxic (RELYEA 2005; BELDEN *et al*. 2010, BRÜHL *et al.* 2013), chronic and delayed effects of sublethal pesticide concentrations on anurans have been found that include prolonged and shortened metamorphosis and reduced body indices (BRIDGES 2000; HOWE *et al*. 2004; CAUBLE & WAGNER 2005; WILLIAMS & SEMLITSCH 2010). Some studies stated different effects if different aquatic developmental stages were exposed to pesticides (GREULICH & PFLUGMACHER 2003; BIGA & BLAUSTEIN 2013). Here, we investigated the effects of two low, environmentally relevant concentrations Focus® Ultra concentration when larvae of the African clawed frog (*Xenopus laevis*) were repeatedly and at different developmental stages exposed. In accordance with other studies on chronic effects of herbicides on anuran larvae (HOWE *et al*. 2004; CAUBLE & WAGNER 2005; WILLIAMS & SEMLITSCH 2010), we hypothesize that repeated exposure will affect mortality, body size and time at metamorphosis. Furthermore, we suppose that early larvae would be more sensitive than later larval stages (BIGA & BLAUSTEIN 2013). Experiments were designed to demonstrate development dependent mortality and time-lag mortality (mortality occurring after cessation of treatment). Endpoints were clinical signs, mortality, body size at metamorphosis, time to metamorphosis and morphological changes.

Material and methods

Test substance

It seems natural that animal studies on toxic effects of insecticides are more numerous than of herbicides (WEIR *et al*. 2012). Lack of data from herbicides and several other reasons mentioned below led to the present study. In the Americas and Europe, corn cultivation has already increased in the last few decades – especially for fodder crop production – but recently, corn cultivation is remarkably expanding for biofuel production (LANDIS *et al*. 2008). While in the Americas and other parts of the world, corn cultivation is mainly based on genetically modified crops with a resistance against the herbicide glyphosate (DUKE & POWLES 2008), in most European countries, genetically modified crop cultivation is only marginally because most of such crops are still undergoing approval (BÖLL *et al*. 2013). Herbicide-resistant corn cultivation is mainly based on cycloxydim-resistant corn hybrids (VANCETOVIC *et al.* 2009). Herbicide-resistant crop cultivation enables repeated applications of the complementary herbicide (not only at beginning of cultivation like in conventional crops), here the cycloxydim-based herbicide formulation Focus® Ultra. This formulation is a selective herbicide containing 100 g/L (= 10.8%) of cycloxydim (CAS 101205-02-1) as active ingredient (a.i.) and 50% of Solvent Naphtha (CAS 64742-94-5) and 4% of sodium dioctylsulphosuccinate (CAS 577-11-7) as added substances. It is also used for foliar spraying against perennial grasses in conventional rape, sugar beet, potato, green bean, and field bean cultivations (EFSA 2012). Its a.i. cycloxydim is a cyclohexene oxime herbicide, i.e. it inhibits acetyl-CoA carboxylase in grasses while dicotyle plants and cycloxdyim-resistant corn hybrids remain unaffected (BURTON *et al*. 1989).

Test organism

The African clawed frog (*Xenopus laevis*) is a pipid anuran amphibian from the southern part of the continent. Developmental biology *of X. laevis* is well known, which was the reason to include the species into the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX, ASTM 1998). Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of both genders. The embryos and the larvae are easy to be kept. The larvae are obligate suspension feeders pumping high amounts of water through their buccopharynx with the suggested consequence of tremendously increased contact to the aquatic environment (VIERTEL 1990, 1992).

Experimental procedure

Based on data from a dose range-finding study, a 96-h LC50 value of 0.1 ± 0.01 mg a.i./L (= 1 mg formulation/L) for early *X. laevis* larvae was selected. From this, we calculated the LC 5 and LC10, which corresponds to rounded 0.01 and 0.05 mg a.i./L (= 0.1 and 0.5 mg formulation/L). Step 1 of the FOCUS (FOrum for the Coordination of pesticide fate models and their USe) surface water models states a worst-case Predicted Environmental Concentration of cycloxydim for surface waters (PECsw; global maximum) of 0.26 mg a.i./L after two applications of 400 g a.i./ha, but in the more realistic FOCUS step 2 and 3 scenarios the PECsw is reduced to 0.003-0.01 mg a.i./L after one application of 600 g a.i./L for shallow bodies of water (EFSA 2012). Hence, the LC5 and LC10 values used as test concentrations represent environmentally relevant concentrations. The experiment was conducted in a climate chamber at Trier University at 23 ± 1 °C. The experimental design included triplicates of controls and four different time points of exposure, which correspond to different application time points in the field.

Tests solutions were freshly prepared using FETAX solution (ASTM 1998). Water was changed weekly, dead animals were removed daily and relevant parameters (oxygen, ammonium, nitrate, pH, hardness and conductivity) were measured before and after water changes. Larvae were fed with 2.5 mg Sera Micron®/animal/day and food rations were doubled after the larvae reached NF stage 51 (“intermediate larval stage”: NIEUWKOOP & FABER 1956). 24-h and 48-h after exposure to Focus® Ultra all individuals were tapped on the tail with a blunt glass rod to assess narcosis by eliciting the flight response. In accordance with MANN & BIDWELL (2001), “mild narcosis” was noted if an individual failed to swim or in an uncoordinated manner, and “full narcosis” if the animal did not respond at all (under a binocular, heart beat revealed that such animals were alive but fully narcotized).

The experiment started eight days after spawning when the larvae from one clutch reached NF stage 47 (“early larvae”). Ten individuals each were kept in full glass aquaria containing 5 L of test solution. Larvae were exposed to 0.01 mg a.i./L (= 0.1 mg formulation/L) and 0.05 mg a.i/L (= 0.5 mg formulation/L) at different developmental stages, i.e. NF stage 47 (“early larvae”), NF stage 51 (“intermediate larval stage” and NF stage 57 (“pre-metamorphosis stage”) and furthermore repeatedly, i.e. at all three stages (see Fig. 1). Control and test concentrations were triplicated. Because water was changed weekly, larvae were exposed to the herbicide for each seven days (corresponds to different application timing and dissipation in the field by microbial degradation, adsorption to sediments etc.) and three times for seven days (repeated exposure; see Fig. 1). We did not exposed groups chronically but repeatedly because this seemed for us a more realistic scenario. Larvae were raised until metamorphosis (NF stage 66). Metamorphs were euthanized by an over-dose of MS-222 (200 mg/L: OECD 2009) and fixed in 5% formaldehyde. 155 days after spawning, the last individuals metamorphosed and the experiment was terminated. Fixed metamorphs were positioned on millimeter paper and were photographed with a digital camera. Snout-vent-length (SVL) was measured using the software “ImageJ” (NATIONAL INSTITUTE OF HEALTH). Differences in mortality, time to metamorphosis and SVL between groups were checked using one-way ANOVA (some data had to be Box-Cox transformed prior to analysis to account for normal distribution and homogeneity of variances), followed by Bonferroni-corrected posthoc-tests (for small sample sizes). The software *R* and the package MASS were applied for statistical analyses (R DEVELOPMENTAL CORE TEAM, Vienna).

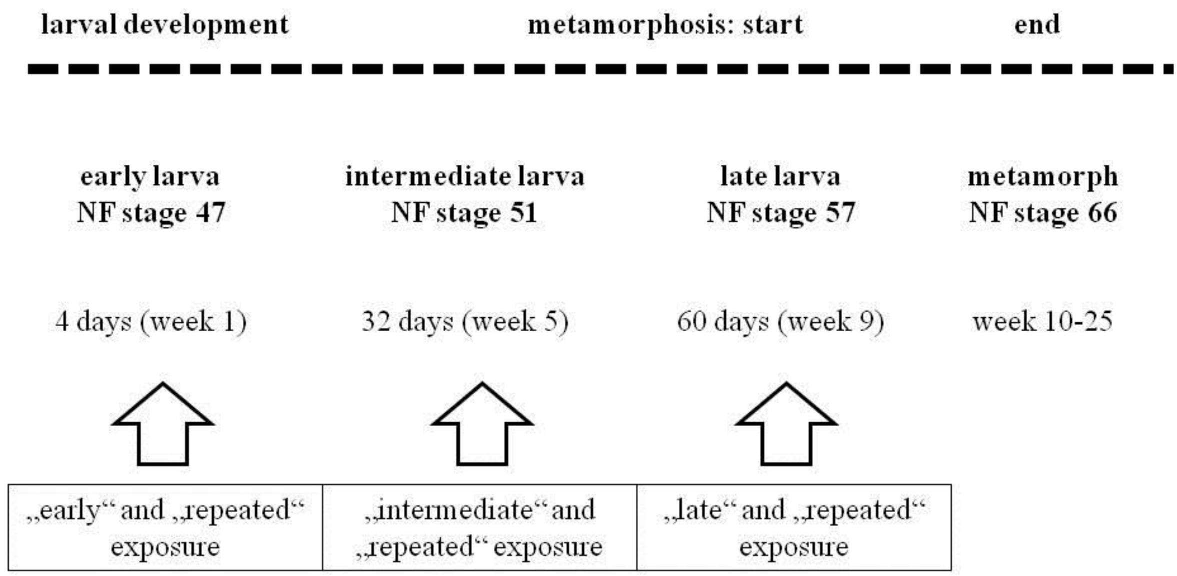

Fig. 1: Exposure scenarios used in the experiment. Arrows indicate exposure timing of different *X. laevis* larval stages to Focus® Ultra. NF stage = developmental stage according to NIEUWKOOP & FABER (1956)

Results and discussion

Dissolved oxygen ranged from 93% to 100%, ammonium from 0 to 0.15 mg/L and nitrate from 0 to 10 mg/L. Average pH value was 7.0, average hardness 6.4 °dH and average conductivity 1472.6 µm/cm. No significant changes in any parameter were observed before or even after any water change demonstrating that the animal density per aquarium and the food amount were not too high and that the weekly water changes contributed to stable water quality and in consequence to the validity of the results.

Narcosis

Exposure to 0.01 mg a.i./L did not result in any clinical signs. Only one early larva showed full narcosis after exposure to 0.05 mg a.i./L. 48-h after exposure to 0.05 mg a.i./L, mild narcosis was similar among all stages (ca. 40%; see Table 1), but 24-h after exposure, mild narcosis increased with the age of the larvae (Table 1) demonstrating increased sensitivity of late larval stages. Furthermore, most of the narcotized larvae recovered (cf. Table 1 and Fig. 2).

Tab. 1: Frequency of mild narcosis of different *X. laevis* larval stages after exposure to 0.05 mg a.i./L (0.5 mg Focus® Ultra/L).

All values ± SE. NF = stages according to NIEUWKOOP & FABER (1956)

Larval stage	24-h after exposure [%]	48-h after exposure [%]
Early larvae (NF 47)	36.6 ± 0.7	40.0 ± 1.0
Intermediate larvae (NF 51)	59.3 ± 1.3	37.1 ± 0.9
Late larvae (NF 57)	72.8 ± 1.2	45.6 ± 0.9

MANN & BIDWELL (2001) observed mild and full narcosis when early larvae of *X. laevis, Rhinella marina, Crinia insignifera, Heleioporus eyrei, Limnodynastes dorsalis* and *Litoria moorei* were exposed to different agricultural surfactants (nonylphenol ethoxylate and alcohol alkoxylate). *X. laevis* larvae were found to be the most sensitive of the tested species. This argues for *X. laevis* to be a good sentinel species in behalf of other anuran larvae due to high sensitivity. Because adverse effects of herbicide formulations are known to be mainly caused by added surfactants and not the active ingredient (WAGNER *et al*. 2013), we suppose one of the added substances of Focus® Ultra responsible for narcotic effects. Also MANN & BIDWELL (2001) observed larvae recovering from narcosis, but stated that (at least) full narcosis would be equivalent to death in the field due to predation. Recovery could be due to (simple) metabolism or the ability of poikilotherms to change the composition of their biological membranes in adaption to varying temperatures and, thereby, adaption to organic chemicals because most narcotic chemicals reduce the phase-transition temperature (MANN & BIDWELL 2001).

Mortality

Exposure to 0.01 mg a.i./L did not result in increased mortality, but time-lag mortality was increased at 0.05 mg a.i./L (Fig. 2). However, the majority of dead early and late larvae were observed only one week after cessation of exposure (see week 2 and week 9 in Fig. 2). This reaction leads to the conclusion that early and late larval stages were more sensitive than intermediate larvae and repeatedly exposed larvae. However, the strong increase in mortality in early larvae only exposed one time and the relatively low mortality in early larvae that were (later on) repeatedly exposed during the experiment (10% vs. >30% mortality; see week 2 in Fig. 2) also demonstrates some biological variance in sensitivity of individuals. Compared to the control, repeated as well as 7 day exposure at all developmental stages significantly increased mortality until end of metamorphosis (ANOVA: $F_{1,13} = 7.73$, $P < 0.05$; Fig. 3). However, no differences in metamorphic success between the treated groups were observed by posthoc tests. Significantly elevated mortality at all developmental stages after exposure to sublethal insecticide (carbaryl) concentrations was also observed in Southern leopard frogs (*Rana sphenocephala*) (BRIDGES 2000). BIGA & BLAUSTEIN (2013) observed species-specific effects on survival when different aquatic life-stages of three Nearctic anurans (*Pseudacris regilla, Rana cascadae* and *R. aurora*) were exposed to sublethal concentrations of another insecticide (cypermethrin). These authors did not observe increased mortality in animals of any species exposed during embryonic development, but in *R. cascadae* when exposed at early and in *P. regilla* when exposed at early and late larval stage.

Body size at and time to metamorphosis

Furthermore, in no case body size at or time to metamorphosis was affected by herbicide treatments. BRIDGES (2000) could observe decreased individual SVL after sublethal insecticide exposure at the egg stage. Likewise, metamorphs of the Moor frog (*Rana arvalis*), which were chronically exposed to another insecticide (α-cypermethrin) (GREULICH &

PFLUGMACHER 2003), metamorphs of four Nearctic anurans (*Rana clamitans, R. pipiens, R. sylvatica* and *Bufo americanus*), which were chronically exposed to glyphosate-based herbicides showed decreased SVL (HOWE *et al.* 2004).

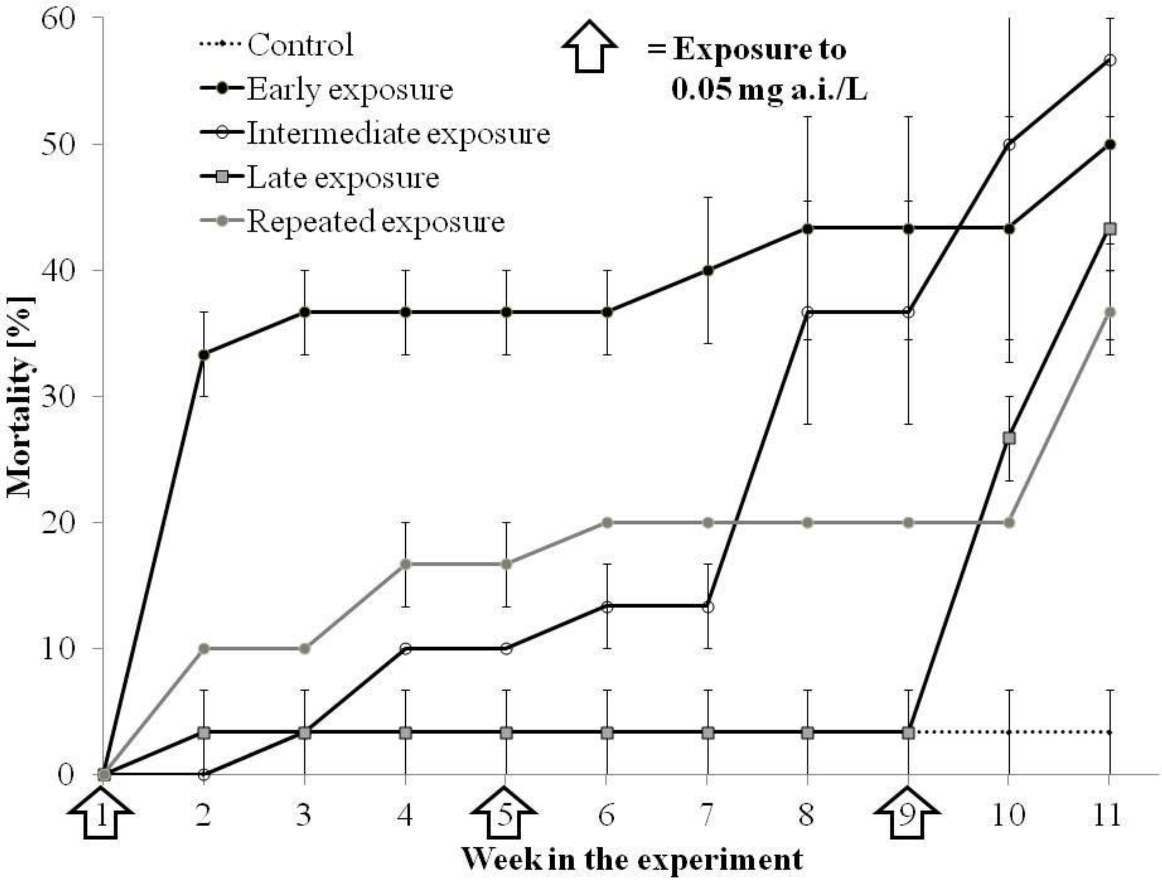

Fig. 2: Time-lag mortality after exposure to 0.05 mg a.i./L (0.5 mg Focus® Ultra/L).

X-Axis according to Fig. 1. Note that the first individuals metamorphosed in week 10 and the last individual in week 25. Week 11 represents the end of metamorphosis for better graphical illustration. From week 1 to 9, the control data are hidden behind the late exposure curve.

Comparable to our results, also BLAUSTEIN & BIGA (2013) did not observe effects on body length when larvae of *Pseudacris regilla, Rana cascadae* and *R. aurora* were exposed to a sublethal insecticide (cypermethrin) concentration at early and late developmental stages. Exposure of eggs and newly hatched individuals of *R. arvalis* to sublethal carbaryl concentrations shortened time to metamorphosis, whereas chronic exposure prolonged time to metamorphosis (GREULICH & PFLUGMACHER 2003). Comparable, chronic exposure to sublethal concentrations of glyphosate-based herbicides led to increased time to metamorphosis in the study by HOWE *et al.* (2004). Only herbicides containing tallowamine surfactants affected development. These authors suggested that disruption of hormone signalling led to effects on the development. There is no evidence that the a.i. (cycloxydim) and the added substances of Focus® Ultra at the low doses under research bear a potential to affect the onset of metamorphosis. It is suggested that the endocrine system inducing and steering metamorphosis remained unimpaired.

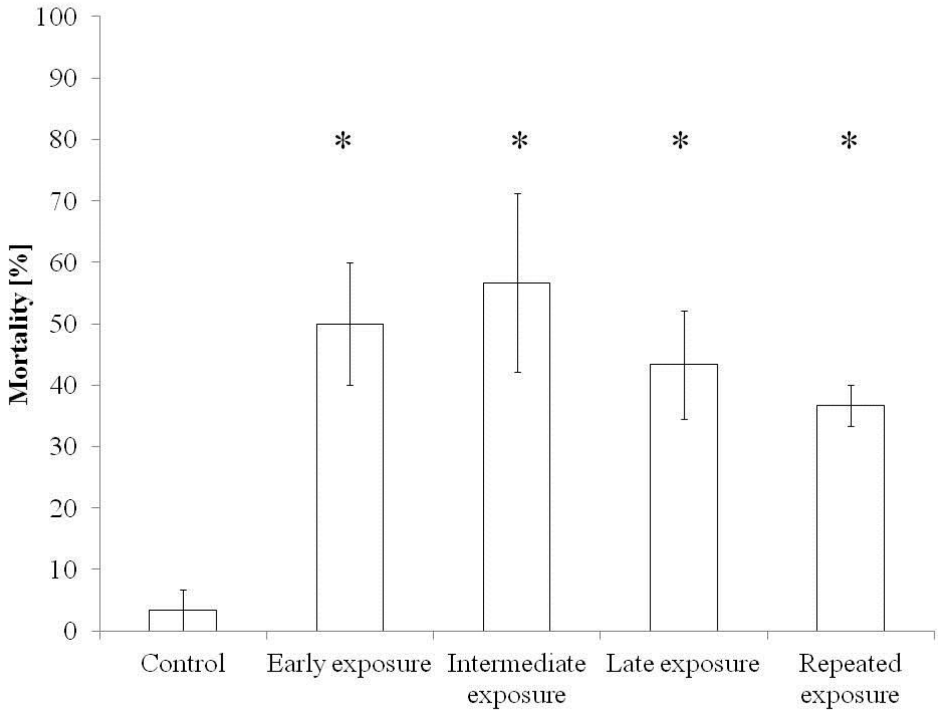

Fig. 3: Increased mortality at metamorphosis of *X. laevis* after exposure to 0.05 mg a.i./L (0.5 mg Focus® Ultra/L).

Asterisks indicate significant differences to the control. All values are given ± standard error.

Morphological changes

Conversely to BRIDGES (2000) and HOWE *et al*. (2004), no malformations could be observed in metamorphs. GREULICH & PFLUGMACHER (2003) observed increased malformation rates in *R. arvalis* larvae (carbaryl exposure) during their experiments. Most individuals did not reach metamorphosis. The authors suggested that the malformations lead to mortality. However, we could only observe one single (most probably spontaneously and not pesticide-induced deformed) *X. laevis* individual with an axial malformation, which died before metamorphosis.

Conclusion

Exposure to low, environmentally relevant doses of Focus® Ultra induced no narcotic or lethal effects at 0.01 mg a.i./L (= calculated LC5). At 0.05 mg a.i./L (= calculated LC10) both was observed narcotic effects in all developmental stages, which may result in increased mortality in the field, and furthermore time-lag mortality. Early larvae were more resistant with regard to narcotic effects than later stages. Metamorphic success was independent of time point and duration of application. The herbicide did not induce effects on body size at and time to metamorphosis or increase deformation rates at all. Hence, the formulation under research seems to affect anuran larvae more acutely than chronically.

Acknowledgments

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Kapitel III: Freilandarbeiten

Are deformation rates of anuran developmental stages suitable indicators for environmental pollution? Possibilities and limitations

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Highlights

- We examined deformations in anurans at different distances to agricultural land.
- More malformed larvae were found in natural wetlands than in agricultural areas.
- Agricultural land use affected other biological parameters in metamorphs.
- 'Unnatural' deformation rates >5% were found in larvae from natural wetlands only.
- The 5%-threshold should be critically evaluated.

Abstract

Among the multitude of reasons identified for amphibian decline, increased use of agrochemicals is suggested to contribute to amphibian population changes in industrialized countries. Contamination of breeding ponds with agrochemicals can provoke developmental effects. Deformation rates of tadpoles and metamorphs thus are expected to be high under agrarian land use. Deformation rates >5% is considered unnatural and implemented in a current amphibian monitoring guideline. We examined deformation rates and different endpoints at metamorphosis in Common frog (*Rana temporaria*) breeding sites (natural wetlands vs. studied waters under potential influence of agrochemicals). Deformation rates were not lower and metamorphosis success was not higher in natural wetlands, but deformation rates >5% were only found here. Rather natural abiotic factors led to higher deformation rates. Our study suggests that the 5%-threshold for unnatural deformation rates should be seen in a critical way until further evaluation if deformation rates of free living amphibians are suitable indicators for environmental pollution.

Keywords: amphibian monitoring, pesticides, *Rana temporaria*, tadpoles, metamorphs, VDI 4333

Introduction

Amphibian populations are globally declining at alarming rates (ALFORD & RICHARDS 1999; HOULAHAN *et al.* 2000; MENDELSON *et al.* 2006; STUART *et al.* 2008). Multiple factors, including tandem effects, are suggested to play a role and the reasons causing declines vary regionally and taxonomically (COLLINS & STORFER 2003; WAKE & VREDENBURG 2008). Destruction and degradation of suitable habitats are severe problems in many regions all over the world (STUART *et al.* 2008), while for instance fungal disease emergence is remarkable for certain taxa and regions only (LA MARCA *et al.* 2005; FISHER *et al.* 2009). In industrialized countries, environmental contamination is supposed to be an important cause for population changes and agricultural activities heavily contribute to it (BOONE *et al.* 2007; STUART *et al.* 2008). Especially, the increasing pesticide use is suggested to have negative effects on biodiversity (GEIGER *et al.* 2010), which in particular is applicable to amphibians (BRÜHL *et al.* 2011). Although proposed, the causality between amphibian declines and agrochemical use remains little studied (MANN *et al.* 2009; WAGNER *et al.* 2013).

Ways of contacts with pesticides include direct and indirect expositions to contaminants, for instance, by direct over-spraying, wind drift or run-off, and all amphibian life stages can be affected (OLDHAM *et al.* 1997; DAVIDSON 2004; DAVIDSON & KNAPP 2007; BRÜHL *et al.* 2011, 2013; BERGER *et al.* 2013). Acute (*e.g.*, RELYEA 2005; SHINN *et al.* 2013), chronic, delayed and indirect (*e.g.*, XU & OLDHAM 1997; HOWE *et al.* 2004; RELEYA 2009; JONES *et al.* 2010, 2011; WILLIAMS & SEMLITSCH 2010; BOONE *et al.* 2013) effects have been reported. Studies are mainly laboratory or mesocosm-based, but also results from *in situ* studies suggest negative impacts of agrochemicals on amphibians (ATTADEMO *et al.* 2007; MCCOY *et al.* 2008; LAJMANOVICH *et al.* 2010).

Various pesticides shape amphibians *in ovo* (ORTON & ROUTLEDGE 2011) and lead to body deformations by teratogenic effects or those appearing during later larval development (BRIDGES 2000; GARDINER *et al.* 2003; LAJMANOVICH *et al.* 2003). But so far, deformation studies mainly aimed at juvenile and adult amphibians and often deformations (especially limb malformations) can be well explained with predation, parasitism and abiotic interactions (BLAUSTEIN & JOHNSON 2003; JOHNSON *et al.* 2003; ANKLEY *et al.* 2004; TAYLOR *et al.* 2005; LANNOO 2008; BALLENGEÉ & SESSIONS 2009). Some observations could be linked to immune suppression due to agrochemicals (KIESECKER 2002; JOHNSON *et al.* 2007; ROHR *et al.* 2008). However, over thirty years after the description of a monitoring on malformations in tadpoles by COOKE (1981), little attention has been given to deformation rates (DRs) of freshly metamorphosed individuals (*e.g.*, VEITH & VIERTEL 1993; PIHA *et al.* 2006) and especially larvae. The studies concluded that a deformation rate (DR) >5% is unnatural.

The 'Association of German Engineers' (VDI) is publishing a series of monitoring handbooks dedicated to detect effects from genetically manipulated crops and their particular agrochemicals on biodiversity. Besides monitoring guidelines for other taxonomic groups like butterflies and moths or soil organisms, the VDI 4333 monitoring guideline aims at amphibians in the agrarian landscape in Austria, Germany and Switzerland. As published in its preliminary version ("Gründruck") (VDI 2013), it uses tadpole DRs according to COOKE (1981) to determine effects potentially associable to herbicides. The guideline foresees 'obligate species' occurring in the agrarian landscape of most of Germany, one of which is the Common frog (*Rana temporaria*) (BÖLL *et al.* 2013).

We applied the procedure described in VDI 4333 with biologically meaningful modifications, to Common frogs in Germany. The purpose was to determine DRs and different parameters at metamorphosis in this species from breeding sites at different distances to agrarian land use. The results of the present work should be incorporated in the final version ("Weißdruck"). As potential alternative explanations to agrochemical use, we recorded several abiotic conditions (water chemistry and climatic variables) at breeding sites.

Our expectations were that (1) DRs and metamorphosis success correlated and that the first mentioned was higher and the second mentioned lower in water bodies in the agrarian landscape. Along with VDI 4333, we expected that (2) DR >5% only occurred at intensively agricultural used sites. Likewise, in accordance with ATTADEMO *et al.* (2014), we expected (3) a correlative decrease in size and mass of metamorphs with distance to agrarian land use. Several studies have shown that agrochemicals can influence the time to metamorphosis, partly by disrupting the thyroid axis in tadpoles (*e.g.*, HOWE *et al.* 2004; WILLIAMS & SEMLITSCH 2010). Hence, we expected that (4) time to metamorphosis differ in tadpoles from agricultural areas and in conspecifics from natural wetlands.

Material and methods

Study sites

Field work was conducted at two localities between March and July 2013 in the Hunsrück low mountain ranges of Rhineland-Palatine, Germany, partly characterized by agricultural land use. The spatial monitoring design of VDI 4333 defines a set of studied waters with different magnitudes of exposure: the three most exposed breeding habitats and three additional, less exposed breeding habitats within a radius of up to 1000 m from the margins of a selected agricultural area (BÖLL *et al.* 2013; VDI 2013). According to this, we chose the available five water bodies (*i.e.*, *R. temporaria* breeding sites) around a high-fertilized hay meadow near the village of Waldweiler (49°36’N, 06°48’ E, about 520 m a.s.l.; Fig. 1) and six breeding sites in the vicinity of a wheat field near the village of Mandern (49°35’N, 06°45’ E, about 470 m a.s.l.; Fig. 2).

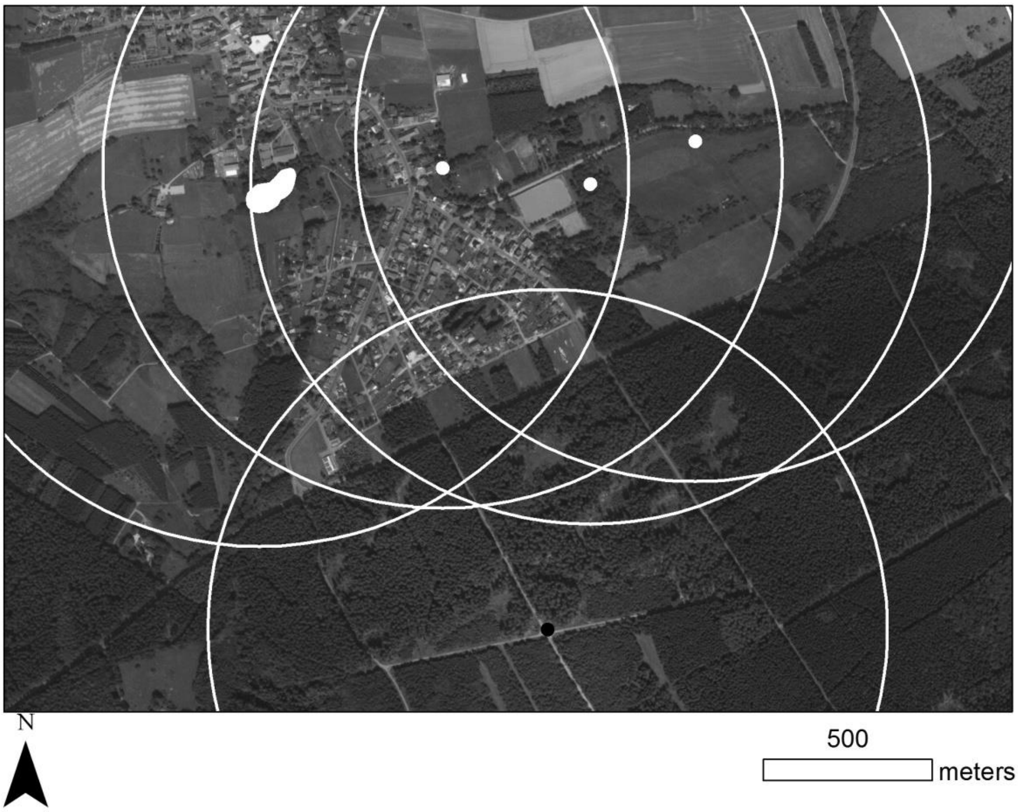

Fig. 1: Breeding sites of *Rana temporaria* around the village of Waldweiler.
White dots indicate small water bodies, which were mainly surrounded by agricultural land use, likewise the white polygon indicates the studied pond situated in the cultivated landscape. The black dot indicates the natural studied water body from this site.

With regard to surrounding land use in general, studied waters were defined as ‘situated in the cultivated landscape’ (‘cultivated studied water bodies’, CSW) when >50% within a 1 km-radius were used for agriculture and settlement. If otherwise, they were defined as control references (‘natural studied water bodies’, NSW). Usually more than 70% of the surrounding land use of NSW was coniferous or mixed forest (Figs. 1+2). ArcMap 10 (Esri®) and current aerial photographs were used to classify land use, as described in VDI 4333. All breeding sites were small and partly temporary water bodies; exceptions were each one CSW and NSW which were ponds (Table 1).

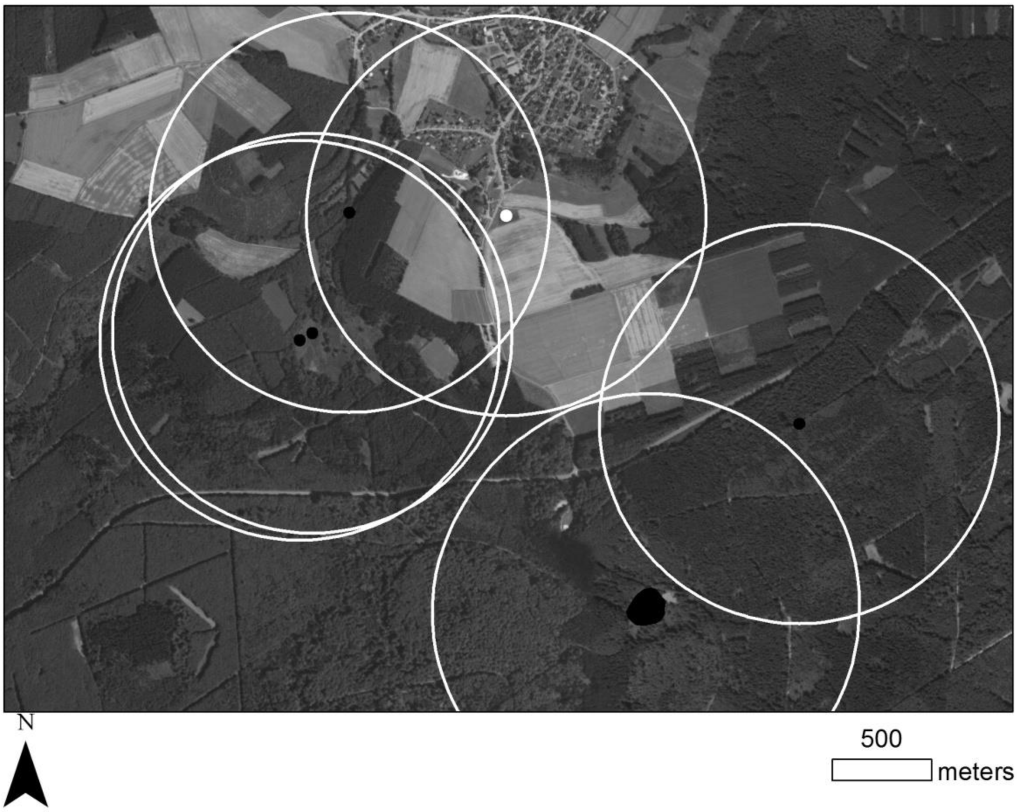

Fig. 2: Breeding sites of *Rana temporaria* around the village of Mandern.
Black dots indicate small, remote water bodies, which were situated in the forest or on a natural wet meadow. Likewise, the black polygon indicates the studied woodland pond. The white dote indicates the studied retention basin, directly adjacent to agrarian land.

Tadpole examination

In accordance with VDI 4333, for DR determination we performed a weekly tadpole monitoring on Common frog larvae. This is a wide-spread species in Germany in which mating takes usually place in February or March, but due to cold weather conditions in 2013, main spawning events of the species in the study region were only at the beginning of April. Several thousands of eggs are laid by one female and various types of water bodies are exploited for tadpole development which lasts into summer (SCHLÜPMANN & GÜNTHER 1996). VDI 4333 foresees a monitoring of early larval stages starting from developmental stage 25 (free swimming tadpoles with formed spiracles) up to 30 (hind limb bud development before toe differentiation starts) employing the developmental staging after GOSNER (1960). For a more complete documentation, we started monitoring with stage 19 (final embryonic stage before animals are defined as hatchlings). From each studied body of water, sixty larvae were randomly sampled every seven days using dip nets. When no specimens of the sampled 60 show deformations, there is a 95% probability that DR is no more than 5% (DIGIACOMO & KOEPSELL 1986) and does not exceed the natural DR. Larvae were euthanized with MS-222 and preserved in 5% formaldehyde. Every individual was carefully examined for presence versus absence of deformations of body and tail following COOKE (1981) and BANTLE *et al.* (1998). However, gut malformations (as described by BANTLE *et al.* 1998) could not be investigated using a binocular because Common frog larvae are more pigmented than *Xenopus laevis* larvae.

According to the VDI 4333, late-stage tadpoles (Gosner stages 36 to 39; final stages of toe differentiation) should not be euthanized and preserved but examined for deformations by photographic documentation for subsequent return into the habitat of origin. Again for more completeness, we documented DRs in Gosner stages 31 to 42 (beginning of metamorphosis). The VDI guideline also defines a weekly sampling of 60 late larval stages per study site when possible, which was not the case throughout the period of our study in some waters. Beside decrease of water level, this was perhaps due to apparently high amounts of tadpole predators.

Tab. 1: Examined breeding sites of *Rana temporaria*.
For temporary water bodies, area and depth were determined until drying out. WW = studied breeding sites from Waldweiler; M = studied breeding sites from Mandern.

Name	Type	Water level	Solar radiation	Area [m²] Ø ± SE	Max. depth [cm] Ø ± SE
Breeding sites situated on or directly adjacent to agricultural land (CSW)					
WW1	Puddle	Temporary	High	2.4 ± 0.5	16.0 ± 2.8
WW2	Pool	Temporary	Low	17.1 ± 3.2	52.5 ± 10.8
WW3	Ditch	Permanent	High	13.8 ± 3.7	75.0 ± 10.2
WW4	Pond	Permanent	High	~ 3,000	~ 500
M1	Puddle	Temporary	High	4.4 ± 0.9	27.5 ± 5.9
Breeding sites situated in the forest (NSW)					
WW5	Ditch	Temporary	High	2.8 ± 0.2	15.0 ± 2.2
M2	Puddle	Temporary	High	3.7 ± 0.7	12.9 ± 2.6
M5	Ditch	Temporary	Low	3.0 ± 1.0	35.0 ± 15.0
M6	Pond	Permanent	Low	~ 7,000	~ 500
Breeding sites situated on natural wet meadow (NSW)					
M3	Pool	Permanent	High	2.9 ± 0.3	15.0 ± 2.7
M4	Pool	Temporary	High	3.9 ± 0.1	16.7 ± 3.3

Metamorph examination

From each study site, as far as applicable, 60 larvae in Gosner stage 42 were collected and brought to Trier University. Specimens were kept in aerated (200 L/h) aquaria containing their home pond water at 23 ± 2°C with one larva per half a liter until metamorphosis was completed. Specific tadpole food (Sera Micron®) was provided *ad libitum*. DRs and metamorphosis success, time to metamorphosis (in days, beginning with the synchronous spawning events in each water body), mass (mg) and snout-vent-length (SVL) (mm) at metamorphosis were taken. Individuals were photographed and SVL was measured using the software ImageJ (NATIONAL INSTITUTE OF HEALTH, Bethesda, USA). Metamorphs were afterwards released at collection sites.

Additional data recording

In accordance with requirements of VDI 4333, the following abiotic co-variables were taken: pH value (directly measured in the field with litmus paper (JBL®)), average water temperature (°C; measured at the beginning and end of sampling with a laboratory thermometer (TFA®)), wind speed and precipitation (each in four classes; see VDI 4333 for details) at the beginning and end of sampling dates and weather condition one day before sampling (1 = rain, 2 = no rain). Rain before sampling is important because maximum concentrations of agrochemicals can usually be found after first rainfalls after application due to run-off or drainage (WAGNER *et al.* 2013). In addition, we measured nitrate, nitrite, chloride, oxygen concentrations (all in mg/L), overall hardness (°dH), carbonate hardness (KH) and conductivity (µS/cm) of water bodies directly in the field with quick tests (JBL®) and conductimeter (WTW®), respectively. Phosphate and ammonium nitrate concentrations (both in mg/L) were measured with JBL® tests immediately after return from sampling (maximum 3 h).

Statistical analyses

DRs of tadpoles from CSW and NSW and among developmental stages were compared using χ^2-tests. Effects of abiotic co-variables on observed deformed individuals were examined using information-theoretic methods (BURNHAM & ANDERSON 2002). Sampling series of <60 individuals were excluded because they had not enough statistical power (DIGIACOMO &

KOEPSELL 1986). Co-variables from the sampling day were considered because deformations in aquatic life stages of anurans (especially embryos and early larvae) occur relatively fast as seen in the use of the 96 h procedure FETAX to assess teratogenic effects of chemicals on *Xenopus laevis* embryos. Also chemical-induced malformations in larvae within 96 h are reported (*e.g.*, YU *et al.* 2013).

We built different candidate models and tested with Generalized Linear Models (GLMs) with log-link for response variables with Poisson distribution (here, deformations) on significant influence ($\alpha = 0.05$) of the predictors and selected the best model, based on small-sample size Akaike Information Criterions (AICc). Candidate models were (1) a 'global model' containing all variables, (2) a 'water chemistry model' containing all water parameters, (3) a 'weather model' containing the climatic parameters only, (4) an 'eutrophication model' only containing nitrate, nitrite, phosphate and ammonium values, (5) a model without auto-correlated variables (tested with a Spearman rank correlation analysis) and (6) a model only containing variables, which explained ≥ 10% of variance (tested with hierarchical partitioning) (Table 2).

Tab. 2: Candidate models for selection using information-theoretic methods.
Effects of co-variables on observed deformed individuals were examined with Poisson GLMs. Sampling series of <60 individuals were excluded.

Model	Co-variables
Model 1 (Global model)	all
Model 2 (Water chemistry model)	nitrate, nitrite, total hardness, carbonate hardness, conductivity, chloride, oxygen
Model 3 (Weather model)	Wind strength, precipitation strength, weather one day before sampling
Model 4 (Eutrophication model)	nitrate, nitrite, phosphate, ammonium
Model 5 (without auto-correlated variables)	nitrate, conductivity, ammonium, pH, average water temperature, weather one day before sampling
Model 6 (only variables explaining >10% of variance)	ammonium nitrate, average water temperature, weather one day before sampling

Possible differences in metamorphosis success between metamorphs from CSW and NSW were examined using χ^2-tests; between time to metamorphosis, SVL and mass of individuals with a one-way ANOVA (for all breeding sites and furthermore grouped in CSW and NSW). Data for time, SVL and mass were Box-cox-transformed. Analyses were conducted with the software *R* and the packages 'hier.part', 'MuMIn' and 'MASS' (R DEVELOPMENTAL CORE TEAM, Vienna).

Results

Tadpoles DRs

In total, 3,367 tadpoles were examined (1,800 from CSW; 1,567 from NSW) with DR of 3.83% (129 malformed individuals). Axial and tail deformations were observed in 115 malformed tadpoles (89.2%) in all developmental stages. Edemas were only observed in 14 (10.8%) early larval stages (Gosner 19-24). Missing parts of the tail (as described in COOKE 1981) or the hindlimbs in larvae were not recorded as deformation in all cases but as trauma-related (*i.e.* apparently predator-induced; see BALLENGEÉ & SESSIONS 2009).

Fifty-one individuals (2.83%) from CSW and 78 (4.98%) from NSW were deformed, *i.e.* significantly more deformed individuals originated from natural wetlands ($\chi^2 = 9.88$; $df = 1$, $P < 0.01$). DRs >5% (abnormal *sensu* COOKE 1981) were found in three NSW only (6.4 – 7.5%; see Fig. 3). Two of these NSW were small ponds, which were situated on a natural wet meadow (M3 and M4; Table 1) and the remaining was a woodland pond (M6). All of these three NSE were surrounded by forest.

No significant differences in the probability to be deformed between developmental stages were found. This was also the case, when different stages from CSW and NSW were compared.

Effects of co-variables on tadpole deformations

Model 6 (only with variables, which explain ≥ 10% of variance) was selected as best model (Table 3). Because models with a ΔAIC ≤ 2 are plausible (BURNHAM & ANDERSON 2002), also model 5 (without auto-correlated variables) should be considered. In both models, higher ammonium values ($Z = 2.0$, $P < 0.05$ and $Z = 2.3$, $P < 0.05$, respectively) and higher water temperature ($Z = 2.6$, $P < 0.01$ and $Z = 2.4$, $P < 0.05$, respectively) significantly increased deformations. Model 6 additionally suggested rain before sampling as an explaining factor ($Z = -2.4$, $P < 0.05$).

Table 4 summarizes the best and second best model.

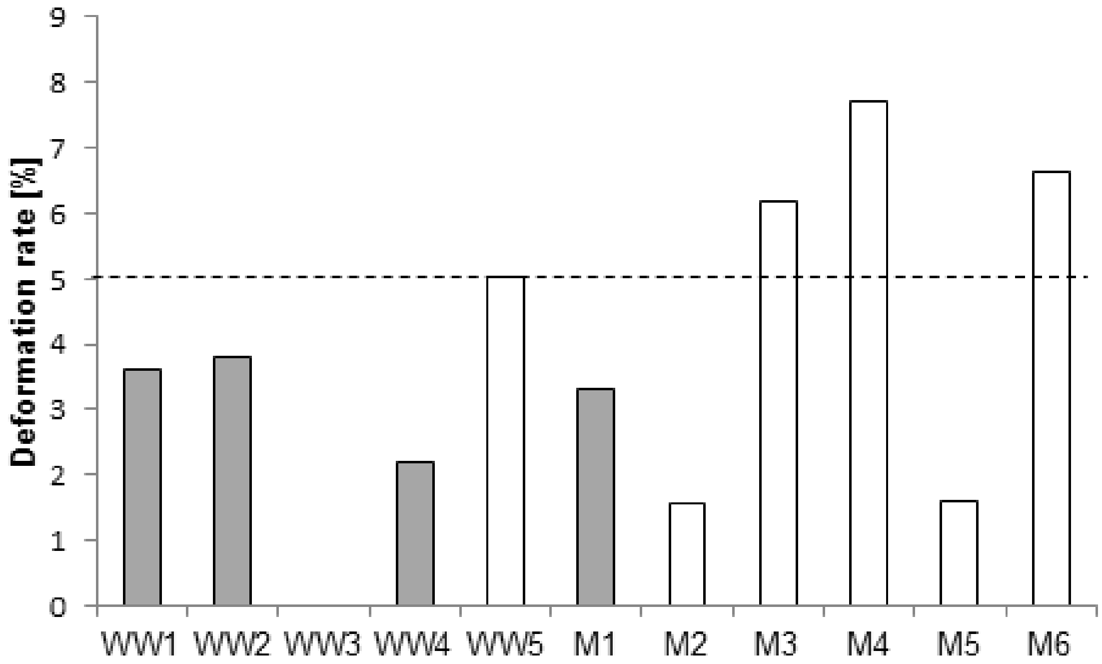

Fig. 3: DRs in the different studied waters.
The dotted line represents the 5%-threshold. WW = studied waters from Waldweiler; M = studied waters from Mandern; dark bars = CSW; light bars = NSW; WW3 is a CSW.

Metamorphs

Deformed metamorphs were recorded only at one site that was directly situated in a high-fertilized hay meadow. Seven out of 51 individuals from this temporary water body showed deformed limbs (13.73%). Micromelia, *i.e.* abnormal smallness of a limb, was observed. Completely and partly missing limbs were found in more individuals (also from other studied waters) but examination using a binocular revealed – as with tadpoles – that these were all trauma-related and rather originated predation stress (*e.g.*, VEITH & VIERTEL 1993). Metamorphosis success was about 50% in average, significantly differed between several ponds (data not shown) but did not differ between individuals from CSW and NSW (Fig. 4A). In addition, individuals from CSW metamorphosed significantly earlier (F = 62.8, df = 1, P <0.001; Fig. 4B), had lower mass (F = 66.8, df = 1, P <0.001; 4C) and were smaller (F = 26.95, df = 1, P <0.001; Fig. 4D) than those from NSW.

Tab. 3: Selection of models using information-theoretic methods.

Model	df	logLikelihood	AICc	ΔAIC	Weight
Model 6	4	-85.4	179.8	0.0	0.5
Model 5	7	-81.3	180.2	0.3	0.5
Model 2	12	-78.1	192.1	12.3	0.0
Model 1	15	-76.5	203.8	24.0	0.0
Model 4	5	-97.2	205.9	26.1	0.0
Model 3	4	-109.5	227.8	48.0	0.0

Tab. 4: Effects of co-variables on DRs in the best and second best model.
SE = standard error of estimate; n.s. = not significant; * = p < 0.05; ** = p < 0.01

	Coefficient	Estimate	SE	*Z*-value	*P*-value
Model 6 (only variables explaining >10% of variance)					
	ammonium nitrate	1.55	0.78	2.00	*
	average water temperature	0.10	0.04	2.58	**
	rainfall before sampling	- 0.54	0.22	- 2.44	*
Model 5 (without auto-correlated variables)					
	nitrate	0.00	0.00	0.11	n.s.
	conductivity	- 0.00	0.00	- 1.64	n.s.
	ammonium nitrate	2.01	0.88	2.29	*
	pH	- 0.23	0.35	- 0.66	n.s.
	average water temperature	0.10	0.04	2.35	*
	rainfall before sampling	- 0.41	0.24	- 1.70	n.s.

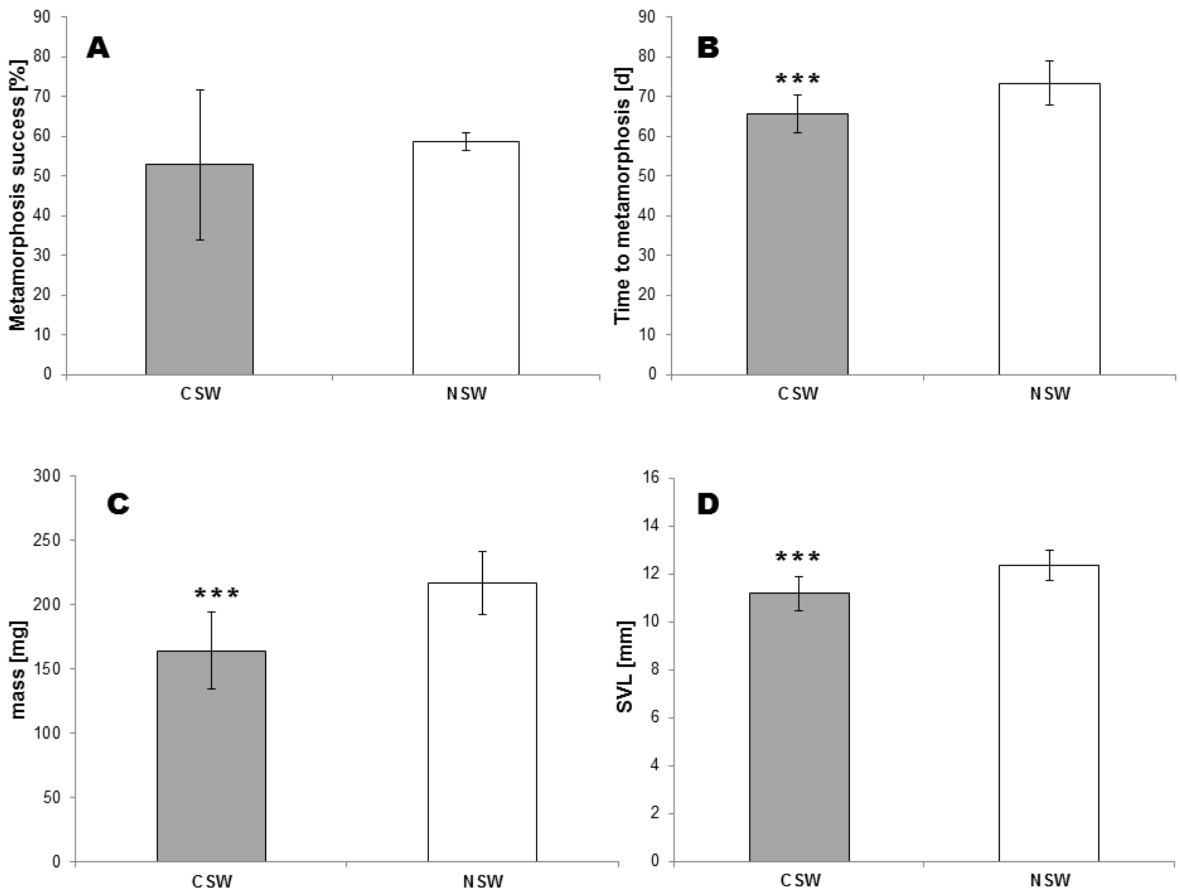

Fig. 4: Average metamorphosis success (A), time to metamorphosis (B), mass (C) and SVL (D) at metamorphosis of individuals from CSW and NSW (± SE).
*** = p < 0.001

Discussion

DR and the 5%-threshold

Based on COOKE (1981), the tadpole monitoring of the VDI 4333 monitoring guideline aims at detecting DR >5%. This was *a priori* expected to be unnatural due to effects of agrochemicals (*i.e.*, by implication, in CSW). In this study, DR >5% could only be observed in NSW, while, on average DR in CSW was <3%. We thus do not support that influence of agrochemicals was high enough in our study area to increase DRs.

The same threshold has also been suggested to apply for metamorphs (PIHA *et al.* 2006), but these authors (performing studies in Finland) found only 1% deformed Common frog metamorphs out of over 4000 individuals. Contra COOKE (1981), our study suggests that 5%-threshold for unnatural DRs should be seen in a critical way. Perhaps this is more an impulse, as at the same time it needs to be taken into account that our work refers to 11 water bodies in the same area only.

Possible explanations for increased tadpole DRs

The results from our work did not indicate that (expected) direct agrochemical use was responsible for highest DRs. GLMs suggested elevated ammonium levels, warmer water and occurrence of rainfall before sampling being responsible for higher DRs.

Ammonium (*e.g.*, JOFRE & KARASOV 1999; OLDHAM *et al.* 1997; ORTIZ-SANTALIESTRA *et al.* 2007) and other nitrogen compounds (*e.g.*, MARCO & BLAUSTEIN 1999; JOHANSSON *et al.* 2001; SHINN *et al.* 2008) are known to affect amphibian health including increased DRs (*e.g.*, SHINN *et al.* 2013). Eutrophication of waters with these compounds can occur due to intensive fertilizer use (MARCO *et al.* 2001; PUNZO & LAW 2006), but eutrophication is also a natural development (*e.g.*, MESAGNE *et al.* 2002). Because the highest DRs of tadpoles in this work were only observed in NSW, elevated ammonium levels might not to be directly human-induced. Indirect anthropogenic impact due to atmospheric deposition of ammonium adsorbed at particulate matter cannot be ruled out (for Germany, see http://www.umweltbundesamt.de/reaktiver-stickstoff-in-umwelt).

Higher water temperatures could be an indicator for small shallow ponds where many adverse substances can accumulate (MANN *et al.* 2003) and where tadpoles were exposed to higher UV-B radiation, which is also known to induce malformations (*e.g.*, BLAUSTEIN *et al.* 1997; BLAUSTEIN & BELDEN 2003). Here again, indirect anthropogenic impact cannot be ruled out because an elevated UV-B radiation is caused by human-induced production of chlorofluorocarbons and other chemicals, which continuously deplete stratospheric ozone (BLAUSTEIN & JOHNSON 2003).

Rainfall before sampling periods should simply wash or drain substances into water bodies (WAGNER *et al.* 2013).

Effects on metamorphs

Here again, no significant effects of agricultural land use on DRs and also on metamorphosis success could be observed. However, our expectation was confirmed that metamorphs from CSW metamorphosed earlier and displayed reduced body size and lower mass compared to conspecifics from NSW. Several agrochemicals are known affect these three parameters by inducing stress (*e.g.*, SMITH 2001; CAUBLE & WAGNER 2005; CASCO *et al.* 2006) but faster metamorphosis resulting in lower mass and smaller body size can also be induced by drying out of small ponds and/or predatory stress (*e.g.*, VAN BUSKIRK & RELYEA 1998). Reduced fitness of metamorphs can lead to higher mortality during the first winter, reduced reproductive potential or prolonged time to first reproduction (SMITH 1987; SEMLITSCH *et al.* 1988; GOATER 1994; ALTWEGG & REYER 2003). However, effects on the population level due to precipitated metamorphosis are poorly understood (WAGNER *et al.* 2013), although recent studies have highlighted the importance of metamorphs on population viability (for an overview see SCHMIDT 2011). Most interestingly, SCHMIDT *et al.* (2012) found that effects of date of and size at metamorphosis of Spadefoot toads (*Pelobates fuscus*) cancelled each other out and animals had equal life-time fitness. Nevertheless, studying the endpoints metamorphosis success, time to and size and mass at metamorphosis could be good first indicators for agricultural impacts on breeding sites, but a site-specific evaluation is necessary to draw causal links.

Conclusions

With regard to our expectations from the beginning, we can conclude that in our study (1) DRs were not higher and metamorphosis success not lower in water bodies in the agrarian landscape. (2) Conversely, highest DRs were found in natural wetlands and DR >5% only occurred here. (3) As we expected, reduced body conditions could be observed for metamorphs from water bodies surrounded by agrarian land use. (4) Likewise, time to metamorphosis differed in tadpoles from agricultural areas and in conspecifics from natural wetlands.

Possibilities and limitations of monitoring DRs

Developmental effects of agrochemicals at environmentally relevant concentrations on aquatic life stages of anurans are found in many laboratory studies (*e.g.*, BURKHART *et al.* 1998; MANN *et al.* 2009 and references therein). For example, the ecotoxicological endpoint 'median teratogenic concentration' (TC50), in the standard procedure FETAX (ASTM 1998) aims at identifying chemicals, which increase DRs in embryos. At specific concentrations, agrochemicals are well known to produce developmental effects in tadpoles (*e.g.*, YU *et al.* 2013). Some studies have identified agrochemicals that resemble retinoid acid and affect the retinoid signal pathway, which could explain these developmental effects (*e.g.*, GARDINER *et al.* 2003; PAGANELLI *et al.* 2010). Likewise, field studies on malformed terrestrial life stages of amphibians from agricultural landscapes are available (for an overview see SESSIONS &

BALLENGEÉ 2010). From this viewpoint, monitoring DRs of anurans is a good indicator for environmental contamination with agrochemicals (*possibility 1*). Malformed metamorphs were very rare in our study and that by PIHA *et al.* (2006). This may allow for the question if tadpoles are the better focus to study deformations. In general, malformed individuals also have decreased survival probabilities, especially due to increased predation risk. For example, MANN & BIDWELL (2001) found narcosis of tadpoles exposed to sublethal concentrations of agricultural surfactants. Likewise, BERNABÒ *et al.* (2011) observed irregular swimming for deformed tadpoles exposed to sublethal concentrations of an insecticide. Hence, exposed animals may be easy prey for predators, even before developmental effects occur. This could be the simple reason, why less malformed individuals (especially metamorphs) can be found in the field than really suffered from agrochemical exposure (*limitation 1*). Information on DRs from the field is sparse. More information on spontaneous frequencies in the field and on tadpole DRs from conventionally agricultural used areas and natural wetlands are necessary to evaluate the 5%-threshold. This threshold for unnatural DRs in anurans should be regarded with some care until a wider data base from the field will be available to eventually evaluate if deformed metamorphs and/or tadpoles of free living amphibians are suitable indicators for environmental pollution (*limitation 2*).

In many agrarian landscapes sufficient or accepted breeding sites of Common frogs (and also other amphibians) are absent, mainly due to on-going intensification of agriculture and destruction and degradation of small waters in cultivated landscapes (SCHLÜPMANN & GÜNTHER 1996). This hampers large-scale monitoring as described in VDI 4333 (*limitation 3*). One possibility could be to cage individuals from nearby waters in present or artificial ponds as performed by COOKE (1981) but also in more recent works (*e.g.*, THOMPSON *et al.* 2004; EDGE *et al.* 2011, 2013). By doing so, animals could be easier sampled and most would not be consumed by predators, so that a sufficient sample size for late larval stages would be ensured (*possibility 2*).

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Effects of water contamination on site selection by amphibians: experiences from an arena approach with European frogs and newts

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Abstract

Pesticide residues in breeding ponds can cause avoidance by at least some amphibian species. So far, outdoor experiments have been performed only with artificial pools in areas where the focus species usually occur and new colonization has been observed. Results of this kind of study are potentially influenced by natural disturbances and therefore are of limited comparability. We used an easily manufactured and standardizable arena approach, in which animals in reproductive condition for some hours had a choice among pools with different concentrations of a contaminant. Because there has been much debate on the potential environmental impacts of glyphosate-based herbicides, we investigated the impact of glyphosate isopropylamine salt (GLY-IS), Roundup LB PLUS (RU-LB-PLUS), and glyphosate's main metabolite aminomethylphosphonic acid (AMPA) on individual residence time in water. The following European amphibian species were tested: Common frog (*Rana temporaria*), Palmate newt (*Lissotriton helveticus*), and Alpine newt (*Ichthyosaura alpestris*). The residence time in water was not significantly affected by concentrations below or slightly above the European Environmental Quality Standards for AMPA or the German ''worst-case'' expected environmental concentrations for GLY-IS and RU-LB-PLUS. Occasionally, microclimatic cofactors (nightly minimum ground temperature, water temperature) apparently influenced the residence time. The major drawback of such quick behavior studies is that results can only be transferred to perception and avoidance of contaminated water but not easily to site selection by amphibians. For example, testing oviposition site selection requires more natural water bodies and more time. Hence, to develop a standard procedure in risk assessment, an intermediate design between an arena approach, as presented here, and previously performed field studies should be tested.

Introduction

Amphibian populations are decreasing at alarming rates worldwide (HOULAHAN *et al.* 2000; STUART *et al.* 2008). Environmental contamination is one of the supposed causes for the observed decreases (BOONE *et al.* 2007; STUART *et al.* 2008). Pesticides are especially suspected to affect freshwater systems, which are exploited by most amphibian species for reproduction and/or part of their annual activity phase (RELYEA & HOVERMAN 2006). Contamination of aquatic but also terrestrial amphibian habitats with pesticides can occur by way of direct over-spraying (RELYEA 2005a; DINEHART *et al.* 2009), drift (DAVIDSON *et al.* 2002, 2004), or runoff (BROWNER 1994). Acute (RELYEA 2005a; RELYEA & JONES 2009) and chronic effects (CAUBLE & WAGNER 2005; HOWE *et al.* 2004) on amphibian health have been reported. However, we currently know little about indirect effects, *e.g.,* how pesticides can alter aquatic site selection (VONESH & BUCK 2007). Chemically contaminated ponds were avoided by some anuran (*i.e.,* frogs and toads) species (TAKAHASHI 2007; VONESH & BUCK 2007). Thereby, one of the supposed causes for amphibian decline, *e.g.,* environmental contamination (BOONE *et al.* 2007), can act simultaneously with others, especially the main problem of habitat loss (STUART *et al.* 2008). Conversely, adults of the species, which apparently are able to perceive pesticides, could protect themselves as well as their offspring from the effects of contaminants (VONESH & KRAUS 2009). Up until now, data on amphibian responses to contaminated water bodies have resulted from field experiments. Artificial ponds were created within the area where the focus species occurs and reproduces, and subsequent colonization (measured by number of eggs per water body) was investigated (TAKAHASHI 2007; VONESH & BUCK 2007; VONESH & KRAUS 2009). Such field studies are effective and useful, but they are of limited comparability because their design must fit the given landscape architecture. Furthermore, they are potentially influenced by nonassessable and unpredictable factors. Apart from this potential uncontrolled disturbance, some aspects that might affect animals' responses remain unknown or are difficult to assess, *e.g.,* population size, sex ratio, etc. Therefore, we tested the effects of contamination on aquatic site selection by amphibians under standardized conditions. Arena approaches can be used to standardize animal behavior experiments. For example, these are widely used in orientation studies in which a defined number of animals can choose among different sources (LANDLER & GOLLMANN 2011). We transferred this design to test residence time of amphibians in water in response to different grades of contamination with a substance, *i.e.,* potential avoidance of contaminated water. We chose three widespread European species as test organisms. Apart from one anuran species, the Common frog (*Rana temporaria*), we included for the first time two newt species: the

Palmate newt (*Lissotriton helveticus*) and the Alpine newt (*Ichthyosaura alpestris*). All tested animals were adults in reproductive condition so they were attracted by water. Hence, experiments took place during the particular breeding time of the test species. The two newts have a prolonged aquatic phase during several months that exceeds their breeding activity. In contrast, the Common frog is an ‘‘explosive breeder,’’ *i.e.*, most adults of a population reproduce within a short time period (see WELLS 1977).

We chose to relate our study to glyphosate-based herbicides (GBHs). These have been suggested to dominate the worldwide herbicide market, and their use is increasing (DUKE & POWLES 2008). Several studies are available on the effects of glyphosate (GLY) and GBHs on anuran embryos (PERKINS *et al.* 2000), larvae (RELYEA & JONES 2009; FUENTES *et al.* 2011), and juvenile or adult animals (MANN & BIDWELL 1999; RELYEA 2005a; DINEHART *et al.* 2009), and they include chronic effects (HOWE *et al.* 2004) and indirect impacts (JONES *et al.* 2010). Interestingly, no studies are available on the effects of GLY’s main metabolite, aminomethylphosphonic acid (AMPA; RUEPPEL *et al.* 1977). In our arena approach with newts, we chose glyphosate isopropylamine salt (GLY-IS), which is the active ingredient of most GBHs (Chemical Abstracts Service [CAS] no. 38641-94-0), and a GBH formulation named Roundup LB PLUS (RU-LB-PLUS), which includes 16 % of an unknown surfactant. Common-frog experiments were performed using AMPA, which is not only the main metabolite of GLY but also of other phosphonate compounds (CAS no. 1066-51-9) (SKARK *et al.* 1998).

Material and Methods

Common frog experiments

Effects of contamination with AMPA on residence time in water were tested for male frogs. Fifty male Common frogs were sampled on March 10 and 11, 2012, when they migrating to their breeding site, a small pond near Trier, Germany. Their reproductive condition was recognized by the presence of black nuptial pads (SCHLÜPMANN & GÜNTHER 1996). Frogs were immediately taken to an experimental site within the area of Trier University. Each 10 male animals were kept in buckets (approximately 1 m in diameter) outdoors until the start of the experiment (the latest one started on March 28). The bottom of the buckets contained humid soil, and pieces of turf served as hiding places. Plenty of fresh water was sprayed daily to prevent dehydration of animals.

AMPA (99.5 % purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared daily with distilled water, and four concentrations were tested: 0, 5, 50, and 500 µg/L. The highest concentration corresponded to AMPA's European Environmental Quality Standard (EQS) for the protection of aquatic biota and against which monitoring data should be assessed. The Water Framework Directive of the European Union (Directive 2000/60/EC) provides a procedure to set EQS. The annual average EQS for AMPA is 450 µg/L. In line with the rounded guide value for surface water proposed by the Swedish authorities, we chose the highest test concentration of 500 µg/L (http://www.egeis.org). To the best of our knowledge, 400 µg/L is the highest environmental concentration that has been reported in water (COUPE *et al.* 2012). Based on the high concentration, we tested two lower concentrations (50 and 5 µg/L). These concentrations are more frequently found in surface waters, including amphibian ponds (STRUGER *et al.* 2008; BATTAGLIN *et al.* 2009). Hence, the tested concentrations are not only legally but also environmentally relevant. Experiments were performed in arenas with 10 replicates/night from March 12 to 28. An arena (1.5 x 1.5 m) was defined by an amphibian drift fence and consisted of a hiding place (leaves and planks) and four artificial pools [plastic pans of 34 x 34 cm, each with a 10-L capacity and a 13-cm maximum depth (Fig. 1)].

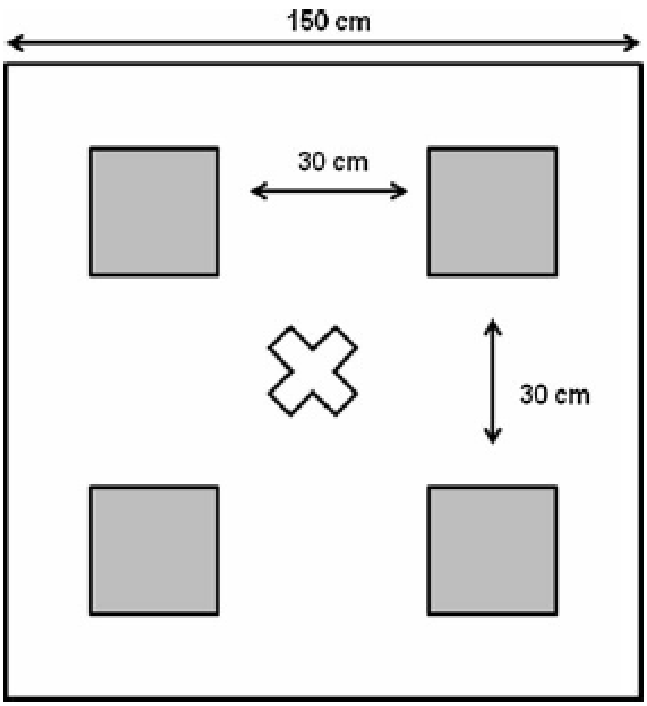

Fig. 1: Composition of one arena (surrounded by an amphibian drift fence).

Gray quadrates indicate the artificial pools, and the cross indicates the animals hiding place.

Arenas were located in a sunny location. This design was chosen per Common frog reproduction behavior, *i.e.*, the size of the breeding water is less important, and shallow sunny water is preferred (*e.g.,* SCHLÜPMANN & GÜNTHER 1996). During heavy rain, a roof protected against decrease of the tested concentrations. The artificial pools contained well water (average pH 7.7, 341 µs/cm, 10.4 mg O_2/L, 8.7°dH). The four concentrations were applied randomly, one to each of the four pools in an arena. Concentrations were renewed every 24 h because AMPA has a half-life of 7–14 days in water depending on local conditions (GIESY *et al.* 2000). In addition, we are aware that AMPA can adsorb to plastic and that plastic can release substances over time. To avoid the influence of conspecifics, site selection was investigated by setting just one male animal into an arena. Because the species is primarily nocturnal, animals were set into the arenas at 19:00. After an adaption period of 1 h, the position of each male animal was recorded every 30 min to 01:00 the following day (*i.e.*, residence time of each individual within 5 h was recorded). After 01:00, the activity in these poikilothermic animals was remarkably decreased or had even stopped. After the experiments, all animals were released to their natural habitat.

Newt experiments

Effects of water contamination with GLY-IS and RU-LBPLUS on residence time were tested with both newt species. A total of each 120 Palmate and Alpine newts were sampled in two ponds near Trier, Germany, using water traps. Sampling was performed between March 29 and April 13, 2012, and always when animals were ‘‘required.’’ Because these sexually dimorphic species are common, sexes could easily be balanced. Study design and methods were the same as described for Common frogs because both newt species are known to accept small water bodies, *e.g.,* cartwheel traces (WINKLER & HEUNISCH 1997). However, newts were set pairwise in each arena (male, female) because it is widely unknown which sex chooses suitable water bodies first or if these animals can perceive the presence of an animal of the other sex in water. Depending on the species, half of the animals (n = 60) were tested with one of the two substances. GLY-IS was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and RU-LB-PLUS was purchased from a local hardware store. In Germany, the estimated worst-case concentration of GLY in surface water after application of the highest approved rate and without buffer strip is approximately 0.9 mg a.e./L (BVL 2010a). Based on this worst-case expected environmental concentration, we used 1 mg a.e./L as a high concentration (again with two lower and more environmentally relevant concentrations of 0.1 and 0.01 mg a.e./L) for both the formulation and the active ingredient.

The annual average EQS for GLY is approximately 100 µg/L (http://www.egeis.org), *i.e.*, our second highest concentration.

Statistical analyses

We used Kolmogorov–Smirnov tests to test for normal distribution of the data. To compare the residence time (min) of individuals in the four groups of contamination, we used Kruskal–Wallis tests followed by Wilcoxon ranksum tests (because these data were not normally distributed). Furthermore, we tested with generalized linear models (GLM) if the different concentrations or considered environmental cofactors influenced the percentage residence time of animals of each trial (*i.e.*, percentage residence time of all individuals tested in 1 night) as proposed for such behavioral studies where uninteresting behavior (here time spent on land) is excluded from analysis (EVERITT & HOWELL 2005). Considered environmental cofactors were average water temperature (as measured with a laboratory thermometer; TFA, Wertheim, Germany), nightly minimum ground temperature, precipitation, relative air humidity (all data were obtained from the weather station Petrisberg, which is situated approximately 1 km airline to the experimental site). Animals that did not enter the water (n = four frogs) were excluded from analysis. The software *R* was used for statistical analyses (R DEVELOPMENTAL CORE TEAM, Vienna).

Results

All frogs and newts survived the experiments, except for one frog, an individual of such poor body condition that we did not link its death to our experiment.

Experiments with Common frogs

On average, male Common frogs stayed on land 54 % of the total observed time, 14.6 % in the control, 10.2 % in the low concentration, 11.8 % in the medium concentration, and 9.4 % in the highest AMPA concentration (Fig. 2). The frogs usually started calling after staying in a pool for more than half an hour, but they also changed pools during observation. On land, they were sitting or migrating most of the time. Some individuals occasionally tried to climb out of the arena, but they usually did not hide. Kruskal–Wallis and the Wilcoxon rank-sum tests on the residence time in the four contamination groups did not show significant differences; likewise, the GLM for percentage residence time did not show a significant influence of any predictor.

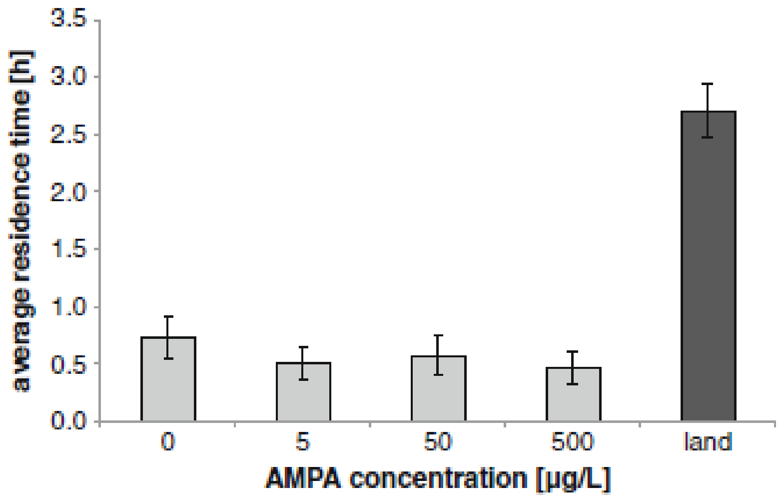

Fig. 2: Average residence time (±SE) of individual male Common frogs (n = 50) in the control, in each of the three AMPA concentrations, and on land.

Newt experiments

Palmate newt

In the experiments with GLY-IS, animals stayed on land 65.2 % of the total observed time, 7 % in the control, 11.2 % in the low concentration, 7.8 % in the medium concentration, and 8.8 % time in the highest GLY-IS concentration (Fig. 3A). On land, animals were hiding most of the time; the rest of the time they were walking on the grass. No animal tried to climb out of the arena. Male animals usually followed female animals into the same pool, but occasionally they were the first to enter the water. After staying several minutes in a pool together, male animals often started courtship behavior. In the experiments with RU-LB-PLUS, Palmate newts stayed on land for 62.1 % of the total observed time, 7.7 % in the control, 12.2 % in the low concentration, 7.3 % in the medium concentration, and 10.7 % in the highest RU-LB-PLUS concentration (Fig. 3B). Animals showed the same behavior on land and in the water as in the previous experiment. For the results of both experiments, Kruskal–Wallis and the Wilcoxon rank-sum tests on the residence time did not show significant differences, and GLM on percentage of residence time did not show significant impacts of any predictor.

Alpine newt

Behavior of Alpine newts was similar to that of Palmate newts. During the experiments with GLY-IS, animals stayed on land for 63.9 % of the total observed time, 11.2 % in the control, 8.7 % in the low concentration, 10.2 % in the medium concentration, and 6 % in the highest

GLY-IS concentration (Fig. 3C). No significant differences between residence time in the four contamination groups were found, but the GLM showed a significantly positive impact of air temperature ($p < 0.001$, $z = 4.53$) and a significantly negative impact of water temperature ($p < 0.05$, $z = -3.11$) on percentage of residence time in the pools. In the experiments with RU-LB-PLUS, newts stayed on land 76 % of the total observed time, 8.5 % in the control, 3 % in the low concentration, 6 % in the medium concentration, and 6.5 % in the highest RU-LB-PLUS concentration (Fig. 3D). Here again, neither Kruskal–Wallis and the Wilcoxon rank-sum tests on residence time nor GLM for percentage of residence time showed significant impacts of any predictor.

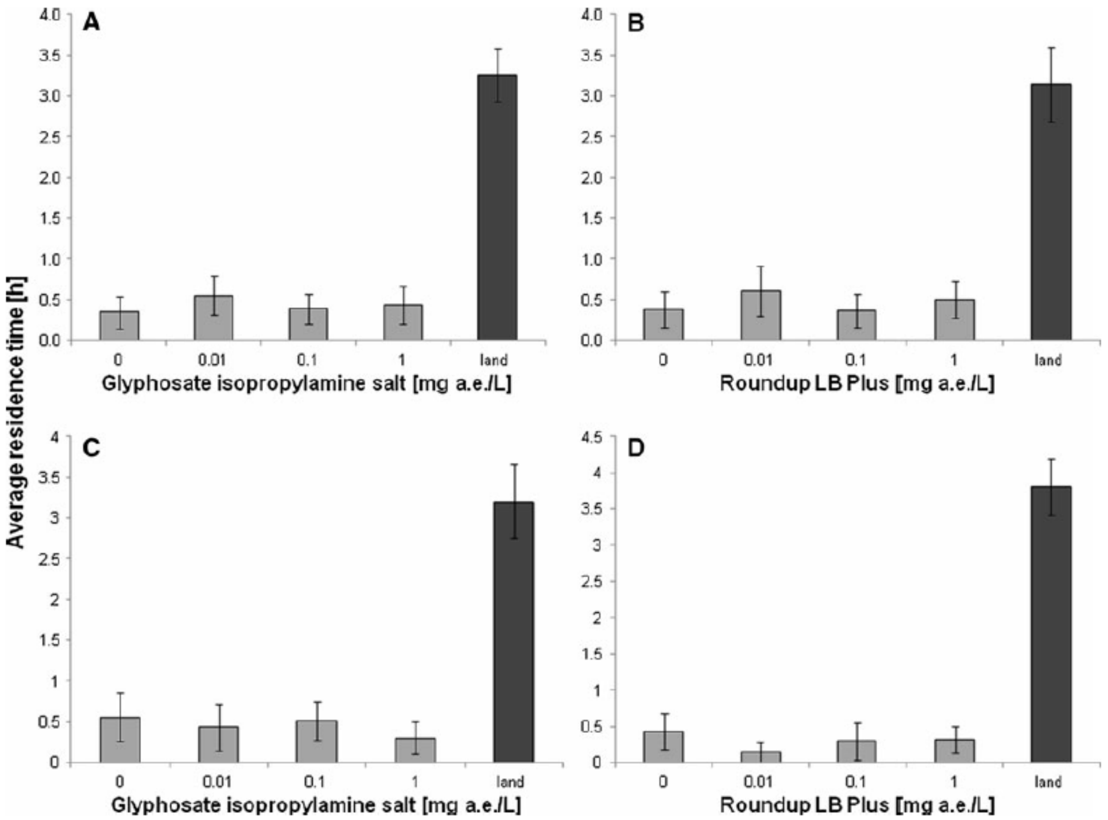

Fig. 3: Average residence time (±SE) of individual Palmate newts (A, B; *n* = 120) and Alpine newts (C, D; *n* = 120) in the control, in each of the three GLY-IS and RU-LB-PLUS concentrations, and on land.

Discussion

Species responses to contaminants

High survival rates were expected because AMPA is considered to be no more toxic than its parent GLY to fish and other standard test organisms (CAREY *et al.* 2008), and LC50 values of diluted GLY on adult anurans are approximately 100 mg a.e./L (MANN & BIDWELL 1999).

Toxicity data of diluted Roundup® formulations on metamorphs and adult amphibians range between 49.4 and 88.7 mg a.e./L (MANN & BIDWELL 1999). Frogs and newts entered artificial pools but remained on land for more than half of the total observed time. In all cases, water contamination with the tested environmentally relevant concentrations did not seem to lead to avoidance of contaminated pools. Conversely, all three species tested in these experiments entered water contaminated at concentrations that slightly exceeded the annual average European EQS (AMPA) or EEC (GLY [Roundup®]). Whether this may cause chronic effects remains to be studied. It is also notable that the surfactant in the Roundup® formulation was either not perceived by the animals or the animals were not bothered by it. In one case, microclimatic cofactors were found to have significant effects on percentage of residence time in newts. This is not surprising because the activity of most amphibians is weatherdependent (DUELLMAN & TRUEB 1986). For example, positive and negative effects of greater air and water temperature could be related to greater activity rates in the newts such that they did not hide on land but entered or changed pools more often. It must be first said that comparability between our study and other amphibian site-selection studies is hampered because in all previous studies artificial ponds were created in known breeding areas. Furthermore, explicitly female responses (*i.e.*, oviposition site selection) were not studied, and male site selection (as studied by us for frogs) and female oviposition site selection are not mandatorily correlated in anurans (RESETARITS & WILBUR 1991). In other anuran site-selection studies, species responded differently to contamination with GBH. North American Gray tree frogs (*Hyla versicolor–chrysoscelis* complex) strictly avoided ponds contaminated with Roundup® (TAKAHASHI 2007). However, only one relatively high concentration (2.4 mg a.e./L) was tested; only five female animals oviposited; and it was not observed if animals entered the contaminated ponds by night time. Both the active ingredient and the formulation of an insecticide (Sevin® and its active ingredient carbaryl, also tested at worst-case scenario concentrations) decreased oviposition site selection by Gray treefrogs compared with water and acetone (solvent) controls (VONESH & BUCK 2007; VONESH & KRAUS 2009). Here, the sample size was large, *i.e.*, >100 female animals accepted the newly created ponds, but again a relatively high concentration was used (7 mg/L). Conversely, no effects of the active ingredient and the formulation on site selection by Northern cricket frogs (*Acris crepitans*) were found by VONESH & KRAUS (2009).

The European newt species tested in our study apparently did not perceive the tested concentrations or did not mind them, but results from GERTZOG *et al.* (2010) suggest that terrestrial Eastern red-backed salamanders (*Plethodon cinereus*) were able to detect a

Roundup® formulation as well as two other herbicides sprayed on soil at approved application rates. It is possible that some amphibians can perceive contamination due to the olfactory sense, which plays a main role in the orientation and communication behavior of most amphibians (DUELLMAN & TRUEB 1986). There is also (indirect) evidence that Wood frogs (*Lithobates sylvaticus*) use chemical cues from predators (fish) for site selection (HOPEY & PETRANKA 1994), which partly support the above-mentioned explanation. Another explanation could be that some species perceive contamination through their permeable skin, so they must enter the water to assess its quality. For example, it has been suggested that amphibians can ''taste'' and examine water with their skin before absorbing it (SMITH *et al.* 2007).

Suitability of the arena approach

It may be taken into account that the studies by TAKAHASHI (2007), by VONESH & BUCK (2007), and by VONESH & KRAUS (2009) not only used different contaminants but also used relatively high concentrations (*e.g.,* estimated after direct applications on the water's surface), whereas our experimental design was based on relatively low but environmentally and legally relevant concentrations. We did not have a ''clear'' positive control – *e.g.,* Roundup® concentrations ≥2.4 mg a.e./L, which were avoided by treefrogs in TAKAHASHI'S (2007) study – to demonstrate. Such a positive control was not possible in our case due to wild animal welfare rights in Germany; furthermore, they may be far from realistic concentrations in most cases. Furthermore, the absence of statistically supported effects could simply be an artifact of the experimental design. For instance, the animals could have been stressed (artifact no. 1); the arenas could have been too small such that animals did not perceive the four pools as distinct water bodies (artifact no. 2); the sample size or scoring procedures could have been inadequate (artifact no. 3); Common frogs have relatively strong home site fidelity, which could have influenced their selection (artifact no. 4); and the used pools were too unnatural (artifact no. 5). Calling activity in frogs and courtship behavior of newts argue for lack of stress, so artifact no. 1 can be disregarded. In addition, artifact no. 5 can disregarded because of our arena approach; however, in a parallel experiment, frog pairs were tested and only 2 of 50 females spawned (data not shown). This could have several reasons, among others (*e.g.,* artifacts no. 4 and 5). Further arena approaches should address artifacts no. 2 and 3, although it may be noted that in terrestrial site–selection studies with different vertebrates, animals were usually smaller or similar arenas were used (*e.g.,* KEEN 1982; SMITH *et al.* 2003). A larger sample size (arenas, nights, and animals) may change the results. This may be appreciated with the help of a power analysis. In our study design (four different concentrations in one replicate), a power analysis was impossible. Because animals stayed longer on land than in the water, a longer observation time during the night would be desirable. Before 20:00 and after 1:00, no or nearly no movement could be observed because all three species are only active at night and are poikilothermic. In summary, it seems necessary to validate the arena approach with further experiments. Perception and avoidance (shown by residence time) of contaminated water bodies should be investigated by arena approaches; however, to study other (relevant) behavior, such as oviposition site selection, more sophisticated designs are needed (at least for some species). The alternative to arena approaches would be mesocosm approaches, field tests (as have already been performed with North American species), or contamination of natal ponds. Contamination of natal ponds would be unethical. Regarding field tests, one could not rely on a sufficiently high sample size for comparability, they are relatively work- and cost intensive, and the performance is difficult in many cases. Hence, mesocosm experiments with (semi)natural ponds, more space, longer time, and more animals should be preferred. In conclusion, assessing the risk of environmental contaminants on habitat selection of amphibians under standardized conditions remains a difficult task.

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Zusammenfassung

Kapitel I: Risikobewertungen

Glyphosatbasierte Herbizide und Amphibien

Die erste in der Einleitung aufgeworfene Fragestellung („*Was ist der aktuelle Wissensstand zu Effekten von Glyphosat und glyphosatbasierten Herbiziden auf Amphibien?*“) wurde in dem vorliegenden Fachartikel (WAGNER *et al.* 2013) aufgegriffen und breit diskutiert. Die wichtigsten Ergebnisse sind, dass – obwohl glyphosatbasierte Formulierungen den weltweit stärksten Absatz besitzen – Daten zu tatsächlicher Umweltbelastung spärlich sind (besonders von den Zusatzstoffen der Herbizide), was wahrscheinlich hauptsächlich mit der teuren Nachweismethodik sowie den relativ geringen Halbwertszeiten der Stoffe im Freiland zusammenhängt. Die kurzen Halbwertszeiten verringern besonders akuttoxische Effekte vieler Formulierungen jedoch nicht, da besonders Mortalität oftmals innerhalb der ersten 24 Stunden beobachtet wurde (etwa RELYEA 2005). Da die Zusatzstoffe (besonders Netzmittel) für den Großteil der bekannten schädlichen Wirkungen auf Amphibien (u.a. Artengruppen) verantwortlich sind, können gemessene als auch geschätzte Umweltkonzentrationen des aktiven Wirkstoffes Glyphosat zudem nur als eine Abschätzung für eine Kontamination mit der jeweils applizierten Formulierung herangezogen werden. Die in den Studien nachgewiesenen Wirkungen auf Amphibien sind abhängig von der Formulierung sowie der betrachteten Amphibienart und dem Lebensstadium. Wie auch bei anderen Umweltchemikalien bereits gezeigt, verstärken weitere abiotische und biotische Stressfaktoren zumeist adverse Effekte.

Zur Beantwortung der zweiten Fragestellung („*Welche Endpunkte sind am besten geeignet, um potenzielle Effekte von glyphosatbasierten Herbiziden und des Wirkstoffes auf Amphibien nachzuweisen?*“) wurden die Aussagen der einzelnen Studien in einer Meta-Analyse ausgewertet. „Effekte auf die Entwicklung von aquatischen Larven“ (etwa Veränderungen in Körpergröße und –masse, Effekte auf den Zeitpunkt der Metamorphose) scheint demnach der sensibelste Endpunkt zu sein, welcher geeignet ist, Effekte von glyphosatbasierten Herbiziden auf Amphibien zu untersuchen.

Bei der derzeitigen Datenlage ist es nicht möglich, einen kausalen Zusammenhang zwischen dem weltweit beobachteten Rückgang von Amphibienpopulationen und dem steigenden Einsatz glyphosatbasierter Herbizide (oder sonstiger Agrochemikalien) zu ziehen (vgl. auch

SCHMIDT 2004; MANN *et al.* 2009). Eine Risikoabschätzung für lokale Amphibienpopulationen sollte immer ortsbezogen und spezifisch erfolgen. Die derzeit publizierten Studien zu Effekten von Herbiziden mit dem Wirkstoff Glyphosat auf Amphibien zeigen zudem, dass mehr Daten zu tatsächlicher Umweltbelastung als auch Amphibienpopulationen in der Kulturlandschaft erhoben werden sollten und dass in Zulassungsverfahren zumindest terrestrische Lebensstadien mit ihrer hochpermeablen Haut spezielle Berücksichtigung finden müssten (vgl. auch QUARANTA *et al.* 2009; BRÜHL *et al*. 2013; BELLANTUONO *et al*. 2014).

Pestizid-Expositionsrisiko von europarechtlich geschützten Amphibien in ihren Schutzgebieten

Die GIS-Analyse in dieser Arbeit (WAGNER *et al.* 2014a) zeigte, dass der proportionale Anteil von Landnutzungsformen, in denen regelmäßig Pestizide appliziert werden, in den SAC der verschiedenen Arten von weniger als 1% bis zu über 40% reicht. Folglich besitzen die Arten alleine auf Grundlage der unterschiedlichen Nutzung ihrer SAC ein unterschiedlich hohes Expositionsrisiko gegenüber Pestiziden. Zudem unterscheidet sich dieses weiterhin durch ihr tatsächliches Vorkommen in diesen landwirtschaftlich genutzten Gebieten und ihrem Lebenszyklus. Durch Literaturrecherche als auch statistische Auswertung konnte herausgefunden werden, dass die meisten Arten, deren Landlebensräume und Laichgewässer häufig in landwirtschaftlich genutzten Gebieten zu finden sind, auch jährliche Wanderungen zu ihren Laichgewässern vollziehen, wo sie größere Ansammlungen bilden. Auf Grundlage ihrer unterschiedlichen Biologie und Ökologie besitzen diese Arten (zumeist „Tieflandamphibien“) ein erhöhtes Expositionsrisiko als eher seßhafte Arten (meist aus höher gelegenen Gebieten). Des Weiteren sind gerade diese Tieflandarten besonders durch Umgestaltung ihrer Lebensräume für die Landwirtschaft betroffen (GALLANT *et al.* 2007), was wiederum einen erhöhten Anteil landwirtschaftlicher Nutzung in ihren SAC bedingt. Zudem konnte aufgezeigt werden, dass sich das Pestizid-Expositionsrisiko für fünf Arten, welche in mehr als einem Mitgliedsstaat vorkommen, in manchen Ländern signifikant voneinander unterscheidet, d.h. die SAC in bestimmten Mitgliedsstaaten besitzen signifikant höhere Anteile landwirtschaftlicher Nutzflächen.

Die meisten Arten mit einem hohen Expositionsrisiko sind auf Grundlage ihres IUCN-Status (noch) nicht in ihrem Gesamtareal gefährdet sind und umgekehrt besitzen weltweit gefährdete Arten sowie prioritäre Anhang II-Amphibienarten meist ein geringes Expositonsrisiko gegenüber Pestiziden. Dies liegt wohl hauptsächlich daran, dass die weltweit gefährdeten Arten eher kleine Areale in Gebirgslagen besitzen oder in anderen abgelegenen Gebieten vorkommen, welche weniger landwirtschaftlich genutzt werden als die Tiefländer, welche wiederum zumeist von den weniger gefährdeten Arten bewohnt werden. Ausnahmen stellen vier Arten dar, welche entweder prioritäre Arten des Natura 2000-Netzwerkes darstellen (*Pelobates fuscus insubricus*) oder in ihren weltweiten Beständen als gefährdet gelten und gleichzeitig Tieflandbewohner sind (*Rana latastei, Triturus dobrogicus, Discoglossus jeanneae*).

Neben einem regelmäßigen Monitoring der Bestände (besonders der Populationen, welche in der Kulturlandschaft vorkommen) sollte auch eines der Habitatkontamination in Managementpläne der SAC aufgenommen werden, um eventuelle pestizidinduzierte Bestandsrückgänge aufzeigen und so eventuell kausale Schlüsse zwischen Pestizideinsätzen und Rückgängen ziehen zu können. Zumindest sollte dies in den SAC für die weltweit gefährdeten Arten mit hohem Pestizid-Expositionsrisiko geschehen, am besten auch in denen aller Tieflandarten. Dies erscheint auch auf Grundlage der unterschiedlichen landwirtschaftlichen Nutzungsintensität in den SAC in verschiedenen Mitgliedsstaaten ratsam.

Kapitel II: Laborexperimente

Im Allgemeinen konnte durch die Laborversuche gezeigt werden, dass das Herbizid Focus® Ultra eine hohe akuttoxische Wirkung auf Embryonen und Larven von *X. laevi*s ausübt, wobei Larven signifikant sensibler als Embryonen reagierten. Nach den Kategorien der USEPA (http://www.epa.gov/oppefed1/ecorisk_ders/ toera_analysis_eco.htm) für die akute Toxizität von Stoffen auf aquatische Organismen kann Focus® Ultra demnach als „*highly toxic*“ für Embryonen (LC50 = 0,1 bis 1,0 mg/L) und „*very highly toxic*“ für Larven von *X. laevis* bezeichnet werden (LC50 < 0,1 mg/L), wenn man den Anteil aktiven Wirkstoffes pro Liter betrachtet. Betrachtet man den Anteil an Formulierung pro Liter, bleibt der Stoff nach USEPA-Kategorien weiterhin „*highly toxic*“ für Larven und „*moderately toxic*“ (LC50 = 1,0 bis 10 mg/L) für Embryonen. Verglichen mit dem einzig verfügbaren Zulassungstest zur Mortalität aquatischer Organismen (durchgeführt mit der Regenbogenforelle, *Oncorhynchus mykiss*), genügt ein zehnfacher Sicherheitsfaktor nur für *Xenopus*-Embryonen und für Larven ist ein hundertfacher Sicherheitsfaktor notwendig. Da für eine Extrapolation zwischen verschiedenen Wirbeltierklassen in der Zulassung ein hundertfacher Sicherheitsfaktor verwendet wird, bestätigen die Ergebnisse (trotz der hohen akuttoxischen Effekte) eher die Ansicht, dass (simple) „klassische" ökotoxikologische Testverfahren zur akuten Toxizität in Verbindung mit Sicherheitsfaktoren genügen, um das akuttoxische Risiko von Focus® Ultra (und wohl den meisten anderen Pflanzenschutzmitteln auch; siehe ALDRICH 2009; WELTJE *et al.* 2013) für aquatische Organismen abzuschätzen. Jedoch ist bekannt, dass in der Natur abiotische und biotische Zusatzstressoren die adversen Effekte von Pestiziden verstärken können, was besonders Mesokosmos-Versuche aufgezeigt haben (siehe hierzu WAGNER *et al.* 2013).

Vergleicht man die Ergebnisse aus den Versuchen mit aquatischen Lebensstadien von *D. scovazzi* – dessen Larven mit denen einheimischer Anuren vergleichbar sind – zeigt sich, dass *X. laevis* gegenüber diesem Nicht-Pipiden signifikant sensibler reagierte, was wohl hauptsächlich durch die bereits angesprochenen unterschiedlichen Pumpraten bedingt ist (VIERTEL 1990, 1992). Auch in den *Discoglossus*-Versuchen waren Larven signifikant sensibler als Embryonen. Die hier beobachtete Mortalität als auch die in *Discoglossus*-Larven nachgewiesene narkotische Wirkung des Herbizids wird wohl ebenfalls durch (simple) „klassische" ökotoxikologische Testverfahren mit aquatischen Standardtestorganismen abgedeckt. Jedoch muss bedacht werden, dass immobilisierte Tiere in der Wildnis wohl oftmals leichte Beute von Prädatoren werden, was die stoffbedingte Mortalität (indirekt) stark erhöhen würde. Zudem verringerte bereits eine Dosis, welche keinerlei Mortalität hervorrief und nur etwa ein Viertel des Wertes betrug, welcher die Hälfte der Tiere narkotisierte (EC50), das Längenwachstum der Tiere signifikant.

Folglich können die ersten drei in diesem Kapitel bearbeiteten Fragestellungen folgendermaßen beantwortet werden: (1) Bezüglich der akuttoxischen Effekte des getesten Herbizids sind „klassische" ökotoxikologische Testverfahren mit aquatischen Standardtestorganismen in Verbindung mit Sicherheitsfaktoren zumeist ausreichend, um das Risiko für Anurenlarven abzuschätzen – zumindest für frühe Entwicklungsstadien der beiden getesteten Arten. Ob solche Ergebnisse aus Laborexperimenten jedoch einfach in die Natur extrapoliert werden können, ist aus oben genannten Gründen fraglich. (2) Wie in anderen Studien zeigte sich auch hier bei beiden Testorganismen, dass Larven signifikant sensibler als Embryonen reagierten, was wohl hauptsächlich dadurch bedingt ist, dass Larven Wasser durch ihren Buccopharynx pumpen und somit verstärkt im Wasser gelösten Stoffen ausgesetzt sind (VIERTEL 1990, 1992; EDGINTON *et al.* 2004). (3) Auch artspezifische Unterschiede in der Sensibilität konnten nachgewiesen werden, was wohl hauptsächlich daran liegt, dass

Xenopus-Larven obligat und *Discoglossus*-Larven nur fakultativ filtrieren (VIERTEL 1990, 1992), was wohl wiederum zu unterschiedlich starkem Kontakt mit der Testsubstanz führte.

In dem Langzeitversuch mit *X. laevis* zeigte sich, dass die verwendeten subletalen Konzentrationen des cycloxydimbasierten Herbizids Focus® Ultra zwar keine Effekte auf Zeit bis und KRL zur Metamorphose sowie die Induktion von Missbildungen besaß – auch unabhängig von dem Zeitpunkt der Exposition. Jedoch starb nach einer viertägigen Exposition gegenüber dem LC10 (welcher den NOEC-Wert für Mortalität darstellte) keine einzige frühe Larve in den *Xenopus*-Versuchen zur akuten Toxizität des Herbizids; diese Konzentration erhöhte aber die Mortalität bis zur Metamorphose in dem Langzeitversuch signifikant – ebenfalls unabhängig vom Zeitpunkt der Exposition. Scheinbar wurde hier Schädigungen der Tiere verursacht, welche sich erst zeitverzögert bemerkbar machten. Hauptaugenmerk bei einer Abschätzung des Umweltrisikos dieses und auch anderer Pflanzenschutzmittels sollte daher besonders auf zeitverzögerte Effekte gelegt werden. Bezüglich der zu Beginn aufgestellten Fragen kann zudem festgehalten werden, dass das Herbizid auf spätere Larvalstadien in stärkerem Maße als auf frühe narkotisierend wirkte, was deren Risiko, im Feld Beute von Prädatoren zu werden, erhöht. Auch hier kann zusammenfassend die Aussage getätigt werden, dass wenn das Umweltrisiko von Pestiziden betrachtet wird, nicht nur reine Laborergebnisse verwendet, sondern diese auch mit natürlichen Umweltbedingungen (etwa der Anwesenheit von Prädatoren) in Verbindung gesetzt werden sollten. Folglich stellen Mesokosmos- aber besonders Freilandarbeiten ein wichtiges Forschungsfeld dar. Nichtsdestotrotz sind die standardisierten Bedingungen in Laborexperimenten keineswegs sinnfrei: nur so können kausale Zusammenhänge zwischen beobachteten Effekten und einer Testsubstanz in einer ersten Risikoabschätzung gezogen werden.

Kapitel III: Freilandarbeiten

Deformationsraten von Anurenlarven

In dieser freilandökologischen Arbeit (WAGNER *et al.* 2014b) stellte sich heraus, dass kein klarer Zusammenhang in der Wahrscheinlichkeit der Ausbildung von Deformationen bei Grasfroschlarven und der die Laichgewässer umgebenden Landnutzung bestand – zumindest in den untersuchten Gewässern. Die höchsten Deformationsraten fanden sich in natürlichen Gewässern. Andere – nicht direkt anthropogen bedingte – Faktoren waren ausschlaggebend: höhere Ammoniumwerte und Wassertemperaturen erklärten in den Modellen die

Wahrscheinlichkeit von Deformationen. Ammonium und andere Stickstoffverbindungen konnten im Labor zu Deformationen bei Anurenlarven führen (SHINN *et al.* 2013); jedoch wurden in Gewässern aus der Agrarlandschaft keine signifikant höheren Werte gemessen als in natürlichen. In den natürlichen Gewässern könnte diese Stickstoffbelastung als Grundlast vorliegen, anthropogen maximal über atmosphärische Exposition bedingt sein (LINDEN 1993). Höhere Wassertemperaturen können für flache Kleingewässer stehen, in denen Larven höherer UV-Strahlung ausgesetzt sind, welche bekannt dafür ist, Deformationen zu verursachen (*e.g.*, BLAUSTEIN *et al.* 1997; BLAUSTEIN & BELDEN 2003). Folglich scheinen Deformationsraten bei Anurenlarven nur bedingt bioindikatorisch für Gewässerbelastung mit Agrochemikalien einsetzbar, d.h. wohl nur bei stärkerer Belastung (besonders teratogen wirkender Substanzen), wobei noch großer Forschungsbedarf zu diesem Thema besteht und diese Freilandstudie keine endgültige Aussage erlaubt.

Klar konnte jedoch in dieser Fallstudie nachgewiesen werden, dass in natürlichen Gewässern (und nur dort) über 5% der Anurenlarven missgebildet waren, so dass der „5%-Schwellenwert“ (siehe COOKE 1981; PIHA *et al.* 2006) keine Allgemeingültigkeit besitzt. Dieses Ergebnis floß in die Endversion der Richtlinie VDI 4333 ein (so dass erst ab einer Missbildungsrate von 5-10% in einem Gewässer neben einer Anbaufläche gentechnisch veränderter Pflanzen genauere Wasser- und Sedimentanalysen durchgeführt werden sollen: VDI 2013).

Obwohl die die Laichgewässer umgebende Landnutzung keinen Einfluss auf den Metamorphoseerfolg hatte, wurde jedoch unsere Vermutung und die Ergebnisse anderer Studien (z.B. BRODEUR *et al*. 2011; ATTADEMO *et al.* 2014) bekräftigt, dass Metamorphlinge aus Gewässern, welche im Agrarland gelegen sind, früher metamorphosierten und zudem kleiner und leichter waren. Ob dies einen spürbaren Einfluss auf der Populationsebene mit sich bringt, kann jedoch nach heutigem Kenntnisstand nicht beantwortet werden, da schlechte Fitnessparameter zwar negativ auf Indivuduen wirken können (SMITH 1987; SEMLITSCH *et al.* 1988; GOATER 1994; ALTWEGG & REYER 2003), bei anderen über die Zeit jedoch kein deutlich negativer Effekt sichtbar wird (SCHMIDT *et al.* 2012). Da die Metamorphlinge auf Grundlage von Populationsmodellen jedoch enorme Wichtigkeit für das Fortbestehen von Amphibienpopulationen besitzen (SCHMIDT 2011), erscheint das Studium der direkten und indirekten Effekte landwirtschaftlicher Tätigkeiten auf biologische Endpunkte bei Metamorphlingen von großem naturschutzfachlichem Interesse (idealerweise gepaart mit der Analyse von Biomarkern: siehe ATTADEMO *et al*. 2014).

Verhaltensversuche

Da es in keinem Fall zu einer signifikanten Meidung kontaminierten Wassers kam, unabhängig welche Substanz und welche Konzentration verwendet wurde, können beide Fragestellungen, welche diesem Verhaltensversuch zugrunde lagen, verneint werden. Inwieweit das Versuchsdesign hierbei eine Rolle spielen könnte, wird in dem Kapitel „*Suitability of the arena approach*“ des Fachartikels (WAGNER & LÖTTERS 2013) ausführlich diskutiert. Die wichtigsten natur- und artenschutzfachlichen Aussagen sind, dass adulte Tiere dieser (und eventuell auch anderer) einheimischen Amphibienarten Gewässer, welche mit umweltrelevanten Konzentrationen der getesteten Substanzen kontaminiert sind, aufsuchen und sich den jeweiligen Konzentrationen aussetzen. Akuttoxische Effekte wurden bei den getesteten umweltrelevanten Konzentrationen nicht beobachtet und auch nicht erwartet, jedoch wurden chronische und verzögerte Effekte auf die exponierten Adulti nicht untersucht. Zudem können aufgrund der höheren Sensibilität aquatischer Lebensstadien Effekte auf diese vermutet werden (WAGNER *et al.* 2013), d.h. wenn Adulti solch kontaminierte Gewässer zur Reproduktion nutzen, kann dadurch der Reproduktionserfolg verringert werden. Da das Versuchsdesign nicht darauf ausgelegt war, kann keine Aussage darüber getroffen werden, ob die Tiere die Gewässer auch zu Reproduktionszwecken nutzen würden, auch weil von männlichen Anuren angenommene Rufgewässer nicht unbedingt mit tatsächlichen Laichgewässern korrelieren müssen (RESETARITS & WILBUR 1991). Von den beiden Molcharten, welche bezüglich der Wahl ihrer Reproduktionsgewässer weniger wählerisch sind und selbst Wagenspuren annehmen (WINKLER & HEUNISCH 1997), kann dies jedoch durchaus vermutet werden. Zudem wurden in diesen Versuchen auch weibliche Tiere verwendet, welche ebenfalls keinerlei signifikante Meidung der Gewässer zeigten. Umgekehrt werden viele zugelassene Pflanzenschutzmittel relativ schnell von Mikroorganismen abgebaut oder adsorbieren an Boden- und Sedimentpartikel, so auch Glyphosat und seine Netzmittel. Folglich können initiale Konzentrationen bei einer einmaligen Belastung des Gewässers während der Laichzeit während der Larvalphase nicht mehr vorhanden sein. Jedoch kann aus den Ergebnissen abgeleitet werden, dass Elterntiere Gewässer aufsuchen, welche potenziell wieder mit diesen und/oder anderen Pestiziden belastet werden können. Letztendlich scheint Gewässerbelastung mit umweltrelevanten Konzentrationen der getesteten Substanzen jedoch umgekehrt nicht zu einer vollständigen Zerstörung bzw. Entwertung dieser aquatischen Amphibienhabitate in landwirtschaftlich genutzten Gebieten zu führen.

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Schlussfolgerungen

In der vorliegenden Dissertationsschrift wurden mehrere Fragestellungen bezüglich des Einflusses von Pestizdieinsätzen auf Amphibien aufgeworfen und mit geeigneten methodischen Ansätzen an diese herangegangen. Abschließend lassen sich auf Grundlage der gewonnenen Ergebnisse kurze Schlussfolgerungen ziehen.

Risikobewertungen

- Selbst für das weltweit am häufigsten verwendete Herbizid Glyphosat sind Daten zu tatsächlicher Umweltbelastung spärlich, besonders von den Zusatzstoffen.

  → Mehr Daten zu tatsächlicher Habitatkontamination als auch Vorkommen von Amphibien in der Kulturlandschaft sind für eine weitere Abschätzung des Umweltrisikos glyphosatbasierter Herbizide notwendig.

- Die Zusatzstoffe (besonders Netzmittel) und nicht der aktive Wirkstoff Glyphosat sind für den Großteil der bekannten schädlichen Wirkungen auf Amphibien (u.a. Artengruppen) verantwortlich. Die in publizierten Studien nachgewiesenen Wirkungen auf Amphibien sind zudem nicht nur abhängig von der Formulierung, sondern auch von der betrachteten Amphibienart und dem Lebensstadium. Wie auch bei anderen Umweltchemikalien bereits gezeigt, verstärken weitere Stressfaktoren (z.B. pH-Wert, Anwesenheit von Prädatoren) zumeist adverse Effekte.

  → Risikobewertungen dieser (als auch anderer Pestizide) sollten sich stets auf die tatsächlich im Feld eingesetzten Formulierungen und nicht nur die aktiven Wirkstoffe beziehen und zudem art- und lebensstadienspezifische Unterschiede als auch in der Natur wirkende abiotische und biotische Faktoren berücksichtigen.

- Der Endpunkt „Effekte auf die Entwicklung von aquatischen Larven" (etwa Veränderungen in Körpergröße und –masse, Effekte auf den Zeitpunkt der Metamorphose) ist der sensibelste Endpunkt, welcher geeignet ist, Effekte von glyphosatbasierten Herbiziden auf Amphibien zu untersuchen.

  → Zukünftige Studien sollten sich besonders auf die Wirkungen subletaler Konzentrationen auf die Entwicklung aquatischer Lebensstadien als auch die zugrunde liegenden Mechanismen (z.B. biochemische Prozesse) konzentrieren.

- In den speziell ausgewiesenen Schutzgebieten für Amphibienarten des Anhangs II der FFH-Richtlinie variiert der Anteil von Landnutzungsformen, in welchen regelmäßig Pestizide appliziert werden, teilweise stark, so dass Arten alleine deshalb ein unterschiedlich hohes Expositionsrisiko gegenüber Pestiziden besitzen. Die meisten Arten, deren Landlebensräume und Laichgewässer häufig in landwirtschaftlich genutzten Gebieten zu finden sind, vollziehen auch jährliche Wanderungen zu ihren Laichgewässern, wo sie größere Ansammlungen bilden. Auf Grundlage ihrer unterschiedlichen Biologie und Ökologie besitzen diese Tieflandarten ein erhöhtes Expositionsrisiko als eher seßhafte Arten. Das Expositionsrisiko kann sich weiterhin zwischen Mitgliedsstaaten signifikant unterscheiden. Die meisten Arten mit einem hohen Expositionsrisiko sind jedoch nicht in ihrem Gesamtareal gefährdet sind. Ausnahmen stellen vier Arten dar.

  → Neben einem regelmäßigen Monitoring der Bestände sollte auch eines der tatsächlichen Habitatkontamination in FFH-Managementpläne aufgenommen werden (auch im Hinblick auf die national unterschiedlichen Expositionsrisiken), um eventuell kausale Rückschlüsse zwischen Bestandsrückgängen und dem Einsatz von Agrochemikalien ziehen zu können. Dabei sollte sich allgemein auf Tieflandarten aufgrund ihres generell hohen Expositionsrisikos konzentriert werden.

Laborxperimente

- Das Herbizid Focus® Ultra besitzt eine eine hohe akuttoxische Wirkung auf Embryonen und Larven von *X. laevi*s, jedoch genügen die Ergebnisse aus „klassischen“ ökotoxikologischen Testsystemen mit aquatischen Standardtestorganismus – in Verbindung Sicherheitsfaktoren – um dieses Risiko abzuschätzen. Auch die letalen Effekte auf frühe Entwicklungsstadien von *D. scovazzi* und die für die Herbizidformulierung nachgewiesene narkotische Wirkung wird durch solche Standardtestverfahren abgedeckt. Jedoch verringerte bereits eine Dosis, welche keinerlei Mortalität hervorrief und nur etwa ein Viertel des Wertes betrug, welcher die Hälfte der Tiere narkotisierte (EC50), das Längenwachstum der Tiere signifikant.

  → Die Endpunkte aus (simplen) „klassischen“ ökotoxikologischen Testsystemen mit aquatischen Standardtestorganismen decken – in Verbindung mit Sicherheitsfaktoren – die Effekte des getesteten Herbizids auf frühe

Entwicklungsstadien der beiden Anuren-Modellorganismen ab, jedoch muss bei einer Abschätzung des Umweltrisikos bedacht werden, dass in der Natur auch weitere abiotische und biotische Stressoren wirken, welche die Effekte verstärken können (etwa dass immobilisierte Tiere in der Wildnis wohl meist leichte Beute von Prädatoren werden).

- *X. laevis* reagierte signifikant sensitiver als der zweite eingesetzte Testorganismus *D. scovazzi*, was wohl hauptsächlich dadurch bedingt ist, dass *Xenopus*-Larven obligat und *Discoglossus*-Larven nur fakultativ filtrieren, was zu unterschiedlich hohen Pumpraten und diese wiederum zu unterschiedlich starkem Kontakt zu im Wasser gelösten Stoffen führt (artspezifische Unterschiede).

  → Die hohe Sensibilität früher Entwicklungsstadien von *X. laevis* spricht für seinen Einsatz als Stellvertreterorganismus für andere Anurenarten. Jedoch muss bedacht werden, dass aquatische Lebensstadien aller einheimischen Anuren denen von *D. scovazzi* ähneln.

- Larven waren immer signifikant sensibler als Embryonen, was wohl hauptsächlich dadurch bedingt ist, dass Larven Wasser durch ihren Buccopharynx pumpen und somit verstärkt im Wasser gelösten Stoffen ausgesetzt sind. Das Herbizid wirkte auf spätere Larvenstadien in stärkerem Maße als auf frühe narkotisierend (lebensstadienspezifische Unterschiede). Dies ist besonders vor dem Hintergrund des Einsatzes von Focus® Ultra beim Anbau cycloxydimresistenter Maishybride zu bedenken, da die Formulierung hier theoretisch über die gesamte Vegetationsperiode appliziert werden kann, wodurch unterschiedliche Entwicklungsstadien von Anuren in Kontakt mit ihm kommen können

  → Applikationen des Herbizids zu unterschiedlichen Zeitpunkten verursachen unterschiedlich starke Effekte.

- In dem Langzeitversuch zeigte sich, dass die verwendeten subletalen Konzentrationen des Herbizids keine Effekte auf Zeit bis und KRL zur Metamorphose sowie die Induktion von Missbildungen besaß. Jedoch erhöhte sich zeitverzögert die Mortalität bis zur Metamorphose signifikant, da scheinbar Schädigungen der Tiere verursacht wurden, welche sich erst zeitverzögert bemerkbar machten.

  → Bei der Abschätzung des Umweltrisikos von Focus® Ultra sollten auch zeitverzögerte toxische Effekte betrachtet werden.

Freilandarbeiten

- Es konnte kein Zusammenhang in der Wahrscheinlichkeit der Ausbildung von Deformationen bei Grasfroschlarven und der die Laichgewässer umgebenden Landnutzung festgestellt werden und die höchsten Deformationsraten fanden sich sogar in natürlichen Gewässern. Andere – nicht direkt anthropogen bedingte – Faktoren waren erklärend. Nur in natürlichen Gewässern konnten über 5% Deformationen bei den Larven nachgewiesen werden.

  → Der „5%-Schwellenwert“ aus der Literatur besitzt keine Allgemeingültigkeit und muss höher angesetzt werden. Prinzipiell sind auf Grundlage von Laborarbeiten Deformationsraten bei Anurenlarven ein geeigneter Endpunkt für die Effekte subletaler Konzentrationen von Agrochemikalien, jedoch sind diese im Feld nur bedingt bioindikatorisch für Gewässerbelastung mit Agrochemikalien einsetzbar (d.h. wohl nur bei starker Belastung).

- Metamorphlinge aus Gewässern, welche im Agrarland gelegen sind, metamorphosierten früher und waren zudem kleiner und leichter. Ob dies einen spürbaren Einfluss auf der Populationsebene mit sich bringt, kann nach heutigem Kenntnisstand zwar nicht klar beantwortet werden, jedoch erscheint das Studium der Effekte landwirtschaftlicher Tätigkeiten auf sie erfolgsversprechend.

  → Effekte auf Entwicklung und morphologische Parameter bei Metamorphlingen scheinen besser geeignet zu sein, um die Auswirkungen geringer Belastung von Gewässern mit Agrochemikalien nachzuweisen.

- Adulti der getesten Arten suchten kontaminierte Gewässer auf und setzten sich den jeweiligen Konzentrationen aus.

  → Gewässerbelastung mit umweltrelevanten Konzentrationen der getesteten Substanzen scheint nicht zu einer vollständigen Zerstörung bzw. Entwertung dieser aquatischen Amphibienhabitate in landwirtschaftlich genutzten Gebieten zu führen.

- Obwohl akuttoxische Effekte auf die exponierten Adulti nicht beobachtet wurden, kann der Reproduktionserfolg aufgrund der erhöhten Sensibilität aquatischer Lebensstadien verringert werden, wenn kontaminierte Gewässer zu Reproduktionszwecken genutzt werden.

→ **Zukünftige Studien sollten die tatsächliche Annahme kontaminierter Gewässer durch einheimische Arten zu Reproduktionszwecken als auch die anschließende Entwicklung des Nachwuchses untersuchen.**

Abschließend lässt sich zusammenfassen, dass – wie bei den meisten wissenschaftlichen Untersuchungen – manche Fragen zwar zufriedenstellend beantwortet wurden, zu manch anderen jedoch noch größerer Forschungsbedarf besteht und durch die Arbeiten auch neue Fragen aufgeworfen wurden. Ich hoffe daher, mit der vorliegenden Arbeit zu den Erkenntnissen der Auswirkungen von Pestizideinsätzen auf Amphibien beigetragen und auch Anstöße zu praktischen Schutzmaßnahmen als auch neuen Forschungsfragen gegeben zu haben.

2. Rechtswissenschaftlicher Teil

Vermeidung schädlicher Stoffeinträge in Fortpflanzungsgewässer von Amphibien durch Gewässerrandstreifenschutz

1. Einleitung

1.1. Gewässerrandstreifen

Gewässerrandstreifen (GR) stellen ein wichtiges naturschutzfachliches Werkzeug dar, um negative anthropogene Einflüsse auf die Wasserqualität sowie die Flora und Fauna in und um Gewässer zu minimieren (NAIMAN *et al.* 2000, S. 996 ff.). Zunächst gehören gewässernahe Habitate wie etwa Auenwälder meistens zu den strukturreichsten und diversesten Lebensräumen in einer Landschaft, die es an sich zu bewahren gilt (GREGORY *et al.* 1991, S. 540 ff.). Die Bewahrung von Binnengewässern mitsamt ihrer Ufer und Auen stellen auch Ziele des Naturschutzes und der Landschaftspflege nach dem BNatSchG[1] dar[2]. Auengebiete und Uferzonen können als semiterrestrische und semiaquatische Übergangsgebiete definiert werden, welche dynamische, heterogene Lebensräume darstellen und daher oftmals Biodiversität auf regionaler bis sogar kontinentaler Skala konzentrieren (DÉCAMPS *et al.* 2010, S. 182). Andere Autoren fanden hingegen keine Hinweise, dass etwa Gebiete um Waldseen verglichen mit weiter entfernten Standorten größeren Artenreichtum und Abundanz an Flora und Fauna beherbergen (MCDONALD *et al.* 2006, S. 1 ff.). In Deutschland und anderen europäischen Ländern gehören etwa noch wenig anthropogen beeinflusste und durch periodische Wasserstandschwankungen geprägte Auengebiete aufgrund der damit einhergehenden zeitlichen als auch räumlichen Heterogenität ihrer Lebensräume zu den artenreichsten Lebensräumen (WARD *et al.* 1999, S. 125) und stellen folglich einen bedeutenden Beitrag zur Erhaltung der nationalen Biodiversität dar (BMU 2007, S. 35 ff.). Diese Gebiete sind die wichtigsten primären Lebensräume für viele einheimische Amphibienarten (KIRSCHEY & WAGNER 2013, S. 283 f.), welche allesamt zumindest

[1] Bundesnaturschutzgesetz vom 29.07.2009 (BGBl. I S. 2542), zuletzt geändert durch Art. 4 Abs. 100 des Gesetzes vom 07.08.2013 (BGBl. I S. 3154).
[2] § 1 Abs. 3 Nr. 3 BNatSchG sowie § 1 Abs. 3 Nr. 5 BNatSchG.

besonderen wenn nicht strengen Schutzstatus genießen[3]. Die deutschen Auengebiete wurden fast vollständig für landwirtschaftliche Nutzung, die Binnenschifffahrt oder den Hochwasserschutz zerstört oder zumindest degradiert (BMU 2007, S. 35 ff.). So können genügend breite GR zunächst als direkter Lebensraum- oder Biotopschutz verstanden werden. Es konnte etwa eine positive Korrelation zwischen der Artenzahl von Brutvögeln und der Breite des verbliebenen flussbegleitenden Waldes beobachtet werden (STAUFFER & BEST 1980, S. 1 ff.). Hier zeigt sich bereits, dass die Breite von GR einen entscheidenden Einfluss auf deren Effektivität besitzt.

Aufgrund ihrer linearen Form stellen Auengebieten und Uferzonen für viele Taxa zudem wichtige Wanderkorridore dar (DÉCAMPS *et al.* 1987, S. 301 ff.; NAIMAN *et al.* 1993, S. 209 ff.; BURBRINK *et al.* 1998, S. 107 ff.). Diese gilt es ebenso zu schützen und ggf. vollständig wiederherzustellen, weshalb Gewässer mitsamt ihrer Randstreifen, Uferzonen und Auen demnach auch im BNatSchG besonders für den Biotopverbund und die Biotopvernetzung hervorgehoben werden[4]. Auch werden Gewässerränder bezüglich ihrer Bedeutung zur Herstellung eines kohärenten Schutzgebietsnetzwerkes im Art. 10 der FFH-Richtlinie[5] namentlich genannt. Jedoch ist die tatsächliche Funktion dieser Gebiete als Wanderkorridor insbesondere für terrestrische Tiere nicht in allen Fällen belegt (NAIMAN & DÉCAMPS 1997, S. 621 ff.). Die notwendige Breite von GR, um die Korridorfunktion zu erhalten, ist zudem schwer abzuschätzen und variiert taxonspezifisch. So wanderten in einer Studie an Flüssen in Vermont die meisten Säugetiere unter oder knapp oberhalb der jährlichen Hochwassermarke, 10-30 m genügten für 90% der Pflanzenarten, welche sich entlang von Flüssen ausbreiten, jedoch wurden 75-175 m für 90% der Vogelarten benötigt (SPACKMAN & HUGHES 1995, S. 325 ff.).

Neben dem direkten Biotopschutz oder als Teil des Biotopverbundes sollen GR jedoch zumeist negative Effekte minimieren, welche sich aus angrenzenden forst- und landwirtschaftlichen Tätigkeiten ergeben. GR in bewaldeten Gebieten sollen die negativen Einflüsse der Holzernte, besonders bei Kahlschlägen, abschwächen, indem sie v.a. einer Verschlechterung der Wasserqualität durch etwa erhöhten Sedimenteintrag oder Änderungen im Temperaturregime entgegenwirken und somit das Leben in den Gewässern schützen (KIFFNEY *et al.* 2003, S. 1060 ff.). Während ohne GR oder mit GR < 30 m Kahlschläge um

[3] vgl. § 7 Abs. 13 und 14 BNatSchG.
[4] § 21 Abs. 5 BNatSchG.
[5] Richtlinie 92/43/EWG des Rates vom 21.05.1992 zur Erhaltung der natürlichen Lebensräume sowie der wildlebenden Tiere und Pflanzen (ABl. L 206, S.7).

Fließgewässer in australischen Eukalyptuswäldern durchweg Makrozoobenthos- und Fischabundanz signifikant verringerten, konnten GR ≥ 30 m diese negativen Effekte kompensieren (DAVIES & NELSON 1994, S. 1289 ff.). Dieser Mindestwert (≥ 30 m) wird auch für kanadische Waldgebiete genannt, um negative Effekte auf Makrozoobenthos und Peripyhton zu verhindern (KIFFNEY *et al.* 2003, S. 1060). Außerdem dienen GR als Rückzugsgebiet für Tiere aus angrenzenden bewirtschafteten oder gar gänzlich entwaldeten Gebieten. Auch hier konnten 30 m Randstreifen um durchfließende Gewässer in Kanada einen signifikanten Verlust im Artenreichtum von Kleinsäugern verhindern (COCKLE & RICHARDSON 2003, S. 133 ff.). Andere Autoren hingegen fanden keine Einflüsse auf Abundanz und Artzusammensetzung von Amphibien und Kleinsäugern nach Kahlschlägen in kanadischen Mischwäldern, wenn GR mindestens 20 m breit angelegt wurden, wiesen aber darauf hin, dass nur Generalisten dieser beiden Artgruppen im Untersuchungsgebiet vorkamen (HANNON *et al.* 2002, S. 1784 ff.). Sehr wohl zeigte sich in dieser Untersuchung jedoch ein negativer Einfluss auf Abundanz und Diversität der dortigen Brutvögel, wenn die GR < 100 m breit waren. Eine Metaanalyse, in welcher 397 Studien zum Nutzen von GR in Waldgebieten als Rückzugsgebiet terrestrischer Organismen ausgewertet wurden, kam zu dem Schluss, dass GR (von 5 bis 200 m) oftmals nicht den gewünschten Erfolg brachten. Besonders stark zeigten sich adverse Effekte bei GR < 50 m. Im Vergleich zu ungestörten Gebieten konnten solche engeren GR nur Effekte auf bestimmte Taxa kompensieren (Vögel, Arthropoden), während andere trotzdem in Abundanz und Artzusammensetzung negativ beeinflusst wurden. Hierzu zählten insbesondere die Amphibien, da diese oftmals ein größeres und heterogeneres terrestrisches Habitat benötigen als in den GR geboten wird (MARCZAK *et al.* 2010, S. 126 ff.). SEMLITSCH & BODIE (2002, S. 1219 ff.) sprechen in ihrer systematischen Literaturübersicht sogar von bis zu 300 m breiten GR, welche für manche Amphibienarten notwendig sind, um einen ausreichend großen terrestrischen Lebensraum für diese zu bieten. Hingegen werden gerade einmal 7-9 m als Kernzone von Amphibienabundanz und -artenreichtum um Quellgewässer in Maine angegeben, welche jedoch ein besonderes Habitat darstellen und damit auch eine besondere Amphibiengemeinschaft beherbergen (PERKINS & HUNTER 2006, S. 2124 ff.). Eine ähnlich enge Kernzone (8 m) wird auch für Salamander genannt, welche sich in kleinen Waldbächen der Appalachen fortpflanzen (PETRANKA & SMITH 2005, S. 443 ff.).

In landwirtschaftlich genutzten Gebieten sollen GR, zusätzlich zu ihrer Funktion als Lebensraum und/oder Korridor, Stoffeinträge durch den Einsatz von Pflanzenschutz- und Düngemitteln abschwächen. Die Effektivität von GR hängt von verschiedenen Faktoren ab.

So konnten 1,52 m breite Randstreifen in Abhängigkeit von den Feuchteverhältnissen in den Randstreifen Eintrittskonzentrationen dreier Herbizide von 100 bis zu nur wenigen Prozenten puffern, wobei sich trockenere Bodenverhältnisse positiv auf die Filterwirkung ausübten (ARORA *et al.* 1996, S. 2160). Starke Regenfälle können die Wirkung negativ, Adsorption im Boden bzw. an Pflanzenmaterial positiv beeinflussen, so dass die Art des Bodens als auch der Bepflanzungsgrad weitere wichtige Faktoren sind (ARORA *et al.* 1996, S. 2161 f.). Auch die Art der Bepflanzung spielt nach Ansicht mehrerer Autoren eine Rolle. So konnten mit Bäumen, Sträuchern als auch Gras bepflanzte Randstreifen Sediment- und Nährstoffkonzentrationen im Abfluss von Feldern um etwa 80% im Vergleich zu Flächen ohne Randstreifen reduzieren (BORIN *et al.* 2005, S. 101). Bestimmte Grassorten (z.B. *Panicum*) können wohl aufgrund ihres dichteren Wurzelsystems im Vergleich zu anderen (z.B. *Festuca*) bessere Ergebnisse in der Filterwirkung erzielen (BLANCO-CANQUI *et al.* 2004, S. 1673). Auch das Alter (bezogen auf das Alter der darauf stockenden Vegetation) von GR spielt eine Rolle, und ältere Randstreifen können mehr potenzielle Stoffeinträge abfiltern als neu angelegte (BORIN *et al.* 2010, S. 103). Jedoch kommen die meisten Studien – neben solchen Faktoren – zum Schluss, dass umso breiter ein Randstreifen angelegt desto effektiver seine Filterwirkung ist (vgl. Tabelle 1). Zudem ist die Filterwirkung natürlich auch stoffspezifisch (Tabelle 1).

Tab. 1: Übersicht zu prominenten Studien zur Filterwirkung verschiedener Randstreifen in Bezug auf P- und N-Düngemittel.

Filterwirkung > 50% und dazugehörige Breite des Randstreifens sind fett gedruckt.

n.g. = nicht genannt

Breite (m)	**Filterwirkung (%)**	**Hangneigung (%)**	**Vegetation**	**Kultur**	**Land**	**Stoff**	**Referenz**
1,5	8	10	Süßgras (*Festuca*)	n.g.	USA	P	DOYLE *et al.* (1977)
4	**62**	10	Süßgras (*Festuca*)	n.g.	USA	P	DOYLE *et al.* (1977)
3,8	**99,7**	10	Bäume	n.g.	USA	P	DOYLE *et al.* (1977)
13,7	**88**	4	Süßgras (*Avena/ Dactylis/ Sorghum*)	Mais, Sommerweizen	USA	P	YOUNG *et al.* (1980)
4,6	39	5-16	Süßgras (*Dactylis*)	n.g.	USA	P	DILLAHA *et al.* (1988)
9,1	**52**	5-16	Süßgras (*Dactylis*)	n.g.	USA	P	DILLAHA *et al.* (1988)
4,6	**75**	5-16	Süßgras (*Dactylis*)	n.g.	USA	P	DILLAHA *et al.* (1988)
9,1	**87**	5-16	Süßgras (*Dactylis*)	n.g.	USA	P	DILLAHA *et al.* (1988)
4,3-5,3	26	n.g.	Süßgras (*Cynodon/ Digitaria*)	n.g.	USA	P	PARSONS *et al.* (1991)
3	40	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	P	LEE *et al.* (1999)
3	35	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	P	LEE *et al.* (1999)

Breite (m)	**Filterwirkung (%)**	**Hangneigung (%)**	**Vegetation**	**Kultur**	**Land**	**Stoff**	**Referenz**
6	**55**	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	P	LEE *et al.* (1999)
6	49	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	P	LEE *et al.* (1999)
2	32	2,3	Süßgras (*Lolium/ Festuca*)	Künstlicher Abfluss	Kanada	P	ABU-ZREIG *et al.* (2003)
5	**54**	2,3-5	Süßgras (*Lolium/ Festuca*)	Künstlicher Abfluss	Kanada	P	ABU-ZREIG *et al.* (2003)
10	**67**	2,3	Süßgras (*Lolium/ Festuca*)	Künstlicher Abfluss	Kanada	P	ABU-ZREIG *et al.* (2003)
15	**79**	2,3	Süßgras (*Lolium/ Festuca*)	Künstlicher Abfluss	Kanada	P	ABU-ZREIG *et al.* (2003)
5	35	2,3	keine	Künstlicher Abfluss	Kanada	P	ABU-ZREIG *et al.* (2003)
6	**81**	1,8	Süßgras (*Festuca*) und Sträucher	Winterweizen, Mais, Soja	Italien	P	BORIN *et al.* (2005)
3	38	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	PO_4^{3-}	LEE *et al.* (1999)
3	30	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	PO_4^{3-}	LEE *et al.* (1999)

Breite (m)	Filterwirkung (%)	Hangneigung (%)	Vegetation	Kultur	Land	Stoff	Referenz
6	46	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	PO_4^{3-}	LEE *et al.* (1999)
6	39	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	PO_4^{3-}	LEE *et al.* (1999)
6	**83**	1,8	Süßgras (*Festuca*) und Sträucher	Winterweizen, Mais, Soja	Italien	PO_4^{3-}	BORIN *et al.* (2005)
3,8	**94,7**	10	Bäume	n.g.	USA	N	DOYLE *et al.* (1977)
13,7	**87**	4	Süßgras (*Avena/ Dactylis/ Sorghum*)	Mais, Sommerweizen	USA	N	YOUNG *et al.* (1980)
4,6	**63**	5-16	Süßgras (*Dactylis*)	n.g.	USA	N	DILLAHA *et al.* (1988)
9,1	**52**	5-16	Süßgras (*Dactylis*)	n.g.	USA	N	DILLAHA *et al.* (1988)
4,6	**61**	5-16	Süßgras (*Dactylis*)	n.g.	USA	N	DILLAHA *et al.* (1988)
9,1	**61**	5-16	Süßgras (*Dactylis*)	n.g.	USA	N	DILLAHA *et al.* (1988)
4,3-5,3	**50**	n.g.	Süßgras (*Cynodon/ Digitaria*)	n.g.	USA	N	PARSONS *et al.* (1991)
3	32	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	N	LEE *et al.* (1999)
3	24	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	N	LEE *et al.* (1999)

Breite (m)	Filterwirkung (%)	Hangneigung (%)	Vegetation	Kultur	Land	Stoff	Referenz
6	**51**	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	N	LEE *et al.* (1999)
6	41	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	N	LEE *et al.* (1999)
6	**78**	1,8	Süßgras (*Festuca*) und Sträucher	Winterweizen, Mais, Soja	Italien	N	BORIN *et al.* (2005)
1,5	**57**	10	Süßgras (*Festuca*)	n.g.	USA	NO_{3-}	DOYLE *et al.* (1977)
4	**68**	10	Süßgras (*Festuca*)	n.g.	USA	NO_{3-}	DOYLE *et al.* (1977)
3	28	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	NO_{3-}	LEE *et al.* (1999)
3	23	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	NO_{3-}	LEE *et al.* (1999)
6	47	n.g.	Standorttypisches Süßgras (*Panicum*)	Künstlicher Abfluss	USA	NO_{3-}	LEE *et al.* (1999)
6	38	n.g.	Süßgras (*Bromus/ Phleum/ Festuca*)	Künstlicher Abfluss	USA	NO_{3-}	LEE *et al.* (1999)
6	**58**	1,8	Süßgras (*Festuca*) und Sträucher	Winterweizen, Mais, Soja	Italien	NO_{3-}	BORIN *et al.* (2005)
4,6	**92**	9	Süßgras (*Poa/ Festuca*)	n.g.	USA	NH_{4+}	BARFIELD *et al.* (1992)

Breite (m)	**Filterwirkung (%)**	**Hangneigung (%)**	**Vegetation**	**Kultur**	**Land**	**Stoff**	**Referenz**
9,1	**100**	9	Süßgras (*Poa/ Festuca*)	n.g.	USA	NH_{4+}	BARFIELD *et al.* (1992)
13,7	**97**	9	Süßgras (*Poa/ Festuca*)	n.g.	USA	NH_{4+}	BARFIELD *et al.* (1992)
6	**63**	1,8	Süßgras (*Festuca*) und Sträucher	Winterweizen, Mais, Soja	Italien	NH_{4+}	BORIN *et al.* (2005)

Phosphor etwa kommt in Düngemitteln in chemischer Verbindung mit Sauerstoff als Phosphat vor und P-Konzentration in den Abflüssen von Agrarflächen betragen bis zu 2 mg/L, obwohl in seltenen Fällen viel höhere Konzentrationen nachgewiesen sind (ABU-ZREIG *et al*. 2003, S. 614). Wenn ein minimaler Randstreifen von 5 m eingehalten wird, sollten P-Konzentrationen durchschnittlich um über die Hälfte reduziert werden (vgl. Tabelle 1). Stickstoff kommt in Form von Nitrat- oder Ammonium auf die Felder. MAYER *et al.* (2007, S. 1172 ff.) führten eine Meta-Analyse zu 45 wissenschaftlichen Studien durch, welche die Filterwirkung von 89 verschieden breiten GR auf Nitrat untersuchten und kamen zu dem Schluss, dass durchschnittlich 4 m genügen, um 50 %, 49 m um 75 % und 149 m um 90 % Nitrat effektiv zu filtern. Diese Angaben stimmen auch größtenteils mit den in Tabelle 1 aufgeführten Werten überein. In Bezug auf Düngemittel ist auch zu beachten, dass P- und N-Düngemittel in Form von Handelsdünger, Wirtschaftsdünger (Gülle, Jauche, etc.) und Klärschlamm durch die landwirtschaftliche Tätigkeit in Gewässer gelangen können, jedoch auch standortspezifisch als natürliche Grundlast vorliegen (LINDEN 1993, S. 5 ff.).

Die Breite von GR spielt also die entscheidende Rolle im Hinblick auf ihre Effektivität, wobei an Maximalzahlen über 50 m als Teillebensraumfunktion für semiaquatische Taxa (MARCZAK *et al.* 2010, S. 126 ff.), etwa 150 m für eine beinahe vollständige Nährstofffilterwirkung (MAYER *et al.* 2007, S. 1173) und fast 200 m Breite zur Funktion als gut funktionierender Wanderkorridor für an Gewässer gebundene Vogelarten (SPACKMAN & HUGHES 1995, S. 325 ff.) genannt werden.

1.2. Rechtlicher Schutzstatus der einheimischen Amphibien und ihr Vorkommen in landwirtschaftlich genutzten Gebieten

Alle einheimischen Amphibienarten sind nach der BArtSchV[6] „besonders geschützt“[7]. Viele in Deutschland vorkommende Amphibien finden sich zudem in den Anhängen II (als Arten von gemeinschaftlichem Interesse, für deren Erhaltung besondere Schutzgebiete ausgewiesen werden müssen) und IV (als Arten für die ein sogenanntes „strenges Schutzsystem“ vorgesehen ist) der FFH-Richtlinie (Tabelle 2). Dieser Schutz umfasst insbesondere die in Art. 12 Abs. 1 lit. a) bis d) der FFH-Richtlinie genannten Verbote, welche in Deutschland in §

[6] Bundesartenschutzverordnung vom 16.02.2005 (BGBl. I S. 258, 896), zuletzt geändert durch Art. 10 des Gesetzes vom 21.01.2013 (BGBl. I S. 95).
[7] § 1 S. 1 BArtSchV i.V.m Anlage 1 Spalte 2.

44 Abs. 1 Nr. 1 bis 4 BNatSchG abgebildet sind („Zugriffsverbote“). Folglich besitzt die Artengruppe der Amphibien einen besonderen artenschutzfachlichen Wert. Dieser hohe rechtliche Schutzstatus ergibt sich besonders daraus, dass die Bestände der meisten Arten sowohl in ihrem gesamten Areal (Tabelle 2), bundesweit als auch in den Ländern zumeist rückläufig sind. Dies spiegelt sich in den Einteilungen in die Kategorien der jeweiligen Roten Listen wider (Tabelle 3), wobei sämtliche Arten nach der „International Union for Conservation of Nature“ (noch) als „Least Concern“ gelten, d.h. nicht in eine IUCN-Kategorie eingeordnet werden, welche ein konkretes Aussterberisiko nahelegt (http://www.iucnredlist.org/initiatives/amphibians).

Tab. 2: In Deutschland vorkommende Amphibienarten (in alphabetischer Reihenfolge), ihr internationaler Populationstrend (nach „International Union for Conservation of Nature" http://www.iucnredlist.org/initiatives/amphibians) und ihr europäischer Schutzstatus (nach FFH-Richtlinie).

Wissenschaftlicher Artname	**Deutscher Artname**	**Internationaler Populationstrend**	**FFH-Richtlinie Anhang II**	**FFH-Richtlinie Anhang IV**
Alytes obstetricans	Geburtshelferkröte	Rückläufig		X
Bombina bombina	Rotbauchunke	Rückläufig	X	X
Bombina variegata	Gelbbauchunke	Rückläufig	X	X
Bufo bufo	Erdkröte	Stabil		
Bufo calamita	Kreuzkröte	Rückläufig		X
Bufo viridis	Wechselkröte	Rückläufig		X
Hyla arborea	Europäischer Laubfrosch	Rückläufig		X
Ichthyosaura alpestris	Bergmolch	Rückläufig		
Lissotriton helveticus	Fadenmolch	Stabil		
Lissotriton vulgaris	Teichmolch	Stabil		
Pelobates fuscus	Knoblauchkröte	Rückläufig		X
Pelophylax kl. *esculentus*	Teichfrosch	Rückläufig		
Pelophylax lessonae	Kleiner Wasserfrosch	Rückläufig		X
Pelophylax ridibundus	Seefrosch	Zunehmend		
Rana arvalis	Moorfrosch	Stabil		X
Rana dalmatina	Springfrosch	Rückläufig		X
Rana temporaria	Grasfrosch	Stabil		
Salamandra atra	Alpensalamander	Rückläufig		X
Salamandra salamandra	Feuersalamander	Rückläufig		
Triturus carnifex	Alpenkammmolch	Rückläufig		X
Triturus cristatus	Nördlicher Kammmolch	Rückläufig	X	X

Der Hauptgrund für die rückläufigen Populationstrends der Amphibien weltweit als auch in Europa und Deutschland liegt in der Zerstörung und Degradierung ihrer Lebensräume (STUART *et al.* 2008, S. 1 ff.). Die dramatischten Rückgänge in Westeuropa fanden in der Zeit nach dem zweiten Weltkrieg statt (HOULAHAN *et al.* 2000, S. 752 ff.) und können mit der damaligen Intensivierung der Landwirtschaft aber auch dem Ausbau der Industrie (i.V.m. einer mangelhaften Umweltrechtslage) in Zusammenhang gebracht werden. Jedoch waren und sind komplexe Interaktionen verschiedener Faktoren am Werk, welche von COLLINS & STORFER (2003, S. 89 ff.) zwei Klassen zugeordnet wurden. Die Hypothesen zum Amphibienrückgang der ersten Klasse beinhalten Faktoren, welche bereits seit über einem Jahrhundert negativ auf die Bestände wirken (invasive Arten, nicht nachhaltige Nutzung, Habitatzerstörung, -degradierung und Landnutzungswandel). Hypothesen der zweiten Klasse werden als rezenter angesehen und wirken wohl hauptsächlich seit der Mitte des letzten Jahrhunderts (Klimawandel und erhöhte UV-B Strahlung, Umweltkontamination, infektiöse Amphibienkrankheiten, v.a. der Amphibien-Chytridpilz). Landwirtschaftliche Nutzung kann folglich mit Hypothesen aus beiden Klassen in Verbindung gesetzt werden: Habitatzerstörung, -degradierung und Landnutzungswandel sowie Umweltkontamination durch den Einsatz von Dünge- und Pflanzenschutzmitteln.

Tab. 3: In Deutschland vorkommende Amphibienarten (in alphabetischer Reihenfolge) und ihr Gefährdungsgrad anhand der Rote Liste Kategorien für Deutschland und die Länder (KÜHNEL *et al.* 2009, S. 1 ff.).

Art	**BRD**	BB	BE	BW	BY	HE	HH	MV	NI	NW	RP	SH	SL	SN	ST	TH
Alytes obstetricans	**3**	-	-	2	1	2	0	-	3	2	3	-	3	-	R	2
Bombina bombina	**2**	2	1	-	-	-	0	2	1	-	-	2	-	2	2	0
Bombina variegata	**2**	-	-	2	2	2	-	-	1	1	2	-	2	0	-	1
Bufo bufo	*****	*	3	V	*	V	*	3	*	*	V	*	*	*	V	*
Bufo calamita	**V**	3	1	2	2	2	1	2	3	3	3	3	2	2	2	2
Bufo viridis	**3**	3	2	2	1	1	0	2	1	2	3	1	3	2	3	1
Hyla arborea	**3**	2	0	2	2	1	1	3	2	2	2	3	1	3	3	3
Ichthyosaura alpestris	*****	2	2	*	*	V	R	-	3	*	V	R	*	*	G	*
Lissotriton helveticus	*****	-	-	*	*	2	R	-	3	*	V	0	*	1	R	V
Lissotriton vulgaris	*****	**	*	V	V	V	V	3	*	*	V	*	V	*	*	*
Pelobates fuscus	**3**	*	2	2	2	1	0	3	3	1	2	3	0	3	*	3
Pelophylax kl. *esculentus*	*****	**	*	D	*	3	D	3	*	*	V	D	*	*	*	*

Art	**BRD**	BB	BE	BW	BY	HE	HH	MV	NI	NW	RP	SH	SL	SN	ST	TH
Pelophylax lessonae	**G**	3	D	G	D	G/D	2	2	2	3	V	D	D	2	D	*
Pelophylax ridibundus	*****	3	*	3	*	G/D	2	2	3	D	2	R	-	3	*	3
Rana arvalis	**3**	*	3	1	1	1	3	3	3	2	1	V	0	3	3	2
Rana dalmatina	*****	R	-	3	3	1	G	1	2	G	2	-	D	3	R	R
Rana temporaria	*****	3	*	V	V	V	V	3	*	*	V	V	*	*	V	V
Salamandra atra	*****	-	-	*	*	-	-	-	-	-	-	-	-	-	-	-
Salamandra salamandra	*****	-	-	3	3	2	0	-	3	*	V	-	*	2	3	3
Triturus carnifex	♦	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-
Triturus cristatus	**V**	3	3	2	2	2	3	2	3	3	2	V	3	2	3	3

BRD = Bundesrepublik Deutschland, BB = Brandenburg, BE = Berlin, BW = Baden-Württemberg, BY = Bayern, HE = Hessen, HH = Hamburg, MV = Mecklenburg-Vorpommern, NI = Niedersachsen und Bremen, NW = Nordrhein-Westfalen, RP = Rheinland-Pfalz, SH = Schleswig-Holstein, SL = Saarland, SN = Sachsen, ST = Sachsen-Anhalt, TH = Thüringen

0 = ausgestorben, 1 = vom Aussterben bedroht, 2 = stark gefährdet, 3 = gefährdet, G = Gefährdung anzunehmen, aber Status unbekannt, R = extrem selten, V = Vorwarnliste, * = ungefährdet, ** = mit Sicherheit ungefährdet, D = Daten unzureichend, - = kein Vorkommen, ♦ = nicht bewertet (da nur allochthone Vorkommen von *T. carnifex* in Bayern)

Die einheimischen Amphibienarten kommen in unterschiedlichen Habitaten vor (GÜNTHER 1996, S. 1 ff.). Arten, welche hauptsächlich an Waldlebensräume und –gewässer gebunden sind (der Alpensalamander ist zudem oftmals auf Almen zu finden), besitzen ein geringes Risiko, dass ihre Lebensräume durch landwirtschaftliche Nutzung degradiert werden. Diese sind in Tabelle 4 in einer Gruppe als „Waldarten“ zusammengefasst. Andere Arten sind eher euryök, d.h. man findet sie sowohl in Wäldern aber auch im Kulturland. Diese besitzen ein höheres Risiko, dass ihre Lebensräume durch die Landwirtschaft in Mitleidenschaft gezogen werden und wurden daher in einer Gruppe als „Euryöke Arten“ zusammengefasst (Tabelle 4). Das höchste Risiko besitzen die Arten, welche hauptsächlich im offenen Kulturland zu finden sind („Offenlandarten“, Tabelle 4). Diese besaßen zumeist ihre Primärhabitate in den heterogenen Lebensräumen der ursprünglichen Wildflussauen und waren durch deren weiträumige Zerstörung (auch für die landwirtschaftliche Nutzung) gezwungen, in Sekundärhabitate auszuweichen, welche oftmals Abbaugebiete darstellen, jedoch auch Landhabitate und Kleingewässer im Kulturland (KIRSCHEY & WAGNER 2013, S. 283 f.). Folglich besitzen die einheimischen Amphibienpopulationen aufgrund ihrer ökologischen Ansprüche ein unterschiedliches Risiko, durch die Landwirtschaft geschädigt zu werden. Solch abweichende Expositionsrisiken gegenüber Agrochemikalien wurde von WAGNER *et al.* (2014, S. 64 ff.) ebenso für weitere europäische Amphibienarten aufgezeigt. Besonders hervorzuheben ist, dass fast alle heimischen Amphibienarten, welche eine aquatische Lebensphase durchlaufen, kleine Gewässer zur Reproduktion bevorzugen (etwa der Grasfrosch und die einheimischen Molcharten) oder oftmals sogar temporäre Kleingewässer wie Pfützen benötigen (etwa die Kreuz- und die Wechselkröte). Diese Kleingewässer sind nach den jeweiligen Wassergesetzen der Bundesländer oftmals als „Gewässer dritter Ordnung“ eingeteilt, während „Gewässer erster und zweiter Ordnung“ große Flüsse und Seen darstellen (siehe Kapitel 2.3), welche von den wenigsten Amphibienarten angenommen werden (vgl. Tabelle 4). Zudem werden länderspezifisch viele Kleingewässer von den Bestimmungen des WHG und des jeweiligen Wassergesetzes des Landes ausgenommen, da sie als „von untergeordneter wasserwirtschaftlicher Bedeutung“ definiert werden (siehe Kapitel 2.3).

Tab. 4: Einteilung der in Deutschland vorkommenden Amphibienarten in Gruppen, welche ihr artspezifisches Risiko darstellen, habitatbedingt durch landwirtschaftliche Nutzung geschädigt zu werden, und Nutzung von Kleingewässern (oftmals als „Gewässer von wasserwirtschaftlich untergeordneter Bedeutung“ definiert) zu Reproduktionszwecken (GÜNTHER 1996, S. 1 ff.).

Wissenschaftlicher Artname	Deutscher Artname	Nutzung von Kleingewässern
	„Waldarten“ (= geringeres Risiko)	
Salamandra atra	Alpensalamander	Nein
Salamandra salamandra	Feuersalamander	Selten
	„Euryöke Arten“ (= mittleres Risiko)	
Alytes obstetricans	Geburtshelferkröte	Ja
Bombina variegata	Gelbbauchunke	Ja
Bufo bufo	Erdkröte	Ja
Ichthyosaura alpestris	Bergmolch	Ja
Lissotriton helveticus	Fadenmolch	Ja
Lissotriton vulgaris	Teichmolch	Ja
Pelophylax kl. *esculentus*	Teichfrosch	Ja
Pelophylax lessonae	Kleiner Wasserfrosch	Ja
Pelophylax ridibundus	Seefrosch	Teilweise
Rana arvalis	Moorfrosch	Ja
Rana dalmatina	Springfrosch	Ja
Rana temporaria	Grasfrosch	Ja
	„Offenlandarten“ (= hohes Risiko)	
Bombina bombina	Rotbauchunke	Ja
Bufo calamita	Kreuzkröte	Ja
Bufo viridis	Wechselkröte	Ja
Hyla arborea	Europäischer Laubfrosch	Ja
Pelobates fuscus	Knoblauchkröte	Ja
Triturus carnifex	Alpenkammmolch	Ja
Triturus cristatus	Nördlicher Kammmolch	Ja

2. Rechtliche Vorgaben zu Gewässerrandstreifen

Rechtliche Vorgaben zu GR finden sich sowohl im Europarecht, im Bundesrecht und in den jeweiligen Wassergesetzen der Bundesländer.

2.1. Europäische Rechtsgrundlage

Die europarechtlichen Grundlagen für den Schutz von Gewässern durch Stoffeinträge durch die Landwirtschaft finden sich in der Wasserrahmenrichtlinie[8] sowie der Nitratrichtlinie[9] und der Pflanzenschutz-Rahmenrichtlinie[10]. Mit dem Inkrafttreten der europäischen WRRL wurde eine europarechtliche Grundlage für eine harmonisierte und integrierte Gewässerschutzpolitik in den Mitgliedstaaten der Europäischen Union geschaffen. In Art. 1 WRRL zeigt sich bereits ihre starke ökologische Ausrichtung. So stellt ein primäres Ziel der WRRL die „Vermeidung einer weiteren Verschlechterung sowie Schutz und Verbesserung des Zustands der aquatischen Ökosysteme und der direkt von ihnen abhängenden Landökosysteme und Feuchtgebiete" dar[11]. Damit werden Uferzonen, Auengebiete als auch durch Stillgewässer geprägte terrestrische Lebensräume in den Schutz mit einbezogen. Laut Art. 1 lit. a) WRRL wird „ein stärkerer Schutz und eine Verbesserung der aquatischen Umwelt, u.a. durch spezifische Maßnahmen zur schrittweisen Reduzierung von Einleitungen, Emissionen und Verlusten von prioritären Stoffen und durch die Beendigung oder schrittweise Einstellung von Einleitungen, Emissionen und Verlusten von prioritären gefährlichen Stoffen" angestrebt. Der aktuell geltenden Anhang X[12] beinhaltet als „prioritäre Stoffe" manche in Deutschland zugelassene Wirkstoffe von PSM wie Isoproturon, während andere „prioritäre" (etwa Atrazin) und auch „gefährliche prioritäre Stoffen" europaweit nicht mehr zugelassen sind (etwa Endosulfan und Trifluralin).

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[8] Richtlinie 2000/60/EG des Europäischen Parlaments und des Rates vom 23.10.2000 (ABl. EU L 327, S. 1) zur Schaffung eines Ordnungsrahmens für Maßnahmen der Gemeinschaft im Bereich der Wasserpolitik.
[9] Richtlinie 91/676/EWG des Rates vom 12.12.1991 (ABl. EG L 37, S. 1) zum Schutz der Gewässer vor Verunreinigung durch Nitrat aus landwirtschaftlichen Quellen.
[10] Richtlinie 2009/128/EG des Europäischen Parlaments und des Rates vom 21.10.2009 (ABl. EU L 309, S. 71) über einen Aktionsrahmen der Gemeinschaft für die nachhaltige Verwendung von Pestiziden.
[11] Art. 1 lit. a) WRRL.
[12] Richtlinie 2013/39/EU des Europäischen Parlaments und des Rates vom 12.08.2013 (ABl. EU L 226, S. 1) zur Änderung der Richtlinien 2000/60/EG und 2008/105/EG in Bezug auf prioritäre Stoffe im Bereich der Wasserpolitik.

Der Europäische Rat bezeichnet in den einleitenden Worten zur Nitratrichtlinie landwirtschaftliche Quellen als Hauptverursacher für die Verschmutzung von Gewässern mit Nitrat. Laut Art. 1 der Nitratrichtlinie besteht deren Ziel darin, „die durch Nitrat aus landwirtschaftlichen Quellen verursachte oder ausgelöste Gewässerverunreinigung zu verringern und weiterer Gewässerverunreinigung dieser Art vorzubeugen“. Die Nitratrichtlinie fordert von den Mitgliedstaaten jedoch zum Zwecke des Gewässerschutzes nur die Aufstellung von „Regeln der guten fachlichen Praxis der Landwirtschaft“, die „von den Landwirten auf freiwilliger Basis anzuwenden sind“[13] (für Deutschland siehe aber Kapitel 2.2).

Die Pflanzenschutz-Rahmenrichtlinie zielt auf die nachhaltige Anwendung von PSM ab und verpflichtet die Mitgliedstaaten, nationale Aktionspläne einzuführen und dabei quantitative Zielvorgaben, Maßnahmen und Zeitpläne zur Verringerung der Risiken für die menschliche Gesundheit und die Umwelt festzulegen[14]. Konkret wird laut Art. 11 Abs. 2 lit. c) Pflanzenschutz-Rahmenrichtlinie der „Einsatz von Risikominderungsmaßnahmen, mit denen das Risiko der Verschmutzung außerhalb der Anwendungsfläche durch Abdrift, Drainageabfluss und Oberflächenabfluss minimiert wird“ gefordert, wozu „die Einrichtung von Pufferzonen in geeigneter Größe zum Schutz der aquatischen Nichtzielorganismen sowie Schutzgebiete für Oberflächengewässer“ gehören.

2.2. Nationale Rechtsgrundlage

Die in Kapitel 2.1. behandelten europäischen Richtlinien wurden in deutsches Recht umgesetzt. Die WRRL wurde im WHG[15] in Bundesrecht umgesetzt. GR dienen demnach „der Erhaltung und Verbesserung der ökologischen Funktionen oberirdischer Gewässer, der Wasserspeicherung, der Sicherung des Wasserabflusses sowie der Verminderung von Stoffeinträgen aus diffusen Quellen“[16]. Der GR umfasst „das Ufer und den Bereich, der an das Gewässer landseits der Linie des Mittelwasserstandes angrenzt“ bzw. berechnet sich „bei

[13] Art. 4 Abs. 1 lit. a) Nitratrichtlinie.
[14] Art. 4 Pflanzenschutz-Rahmenrichtlinie.
[15] Wasserhaushaltsgesetz vom 31.07.2009 (BGBl. I S. 2585), zuletzt geändert durch Art. 4 Abs. 76 des Gesetzes vom 07.08.2013 (BGBl. I S. 3154).
[16] § 38 Abs. 1 WHG.

Gewässern mit ausgeprägter Böschungskante ab der Böschungsoberkante“[17]. Allgemein wird eine bundesweite GR-Breite von 5 m für den Außenbereich (in welchem landwirtschaftlich genutzte Gebiete liegen) vorgeschrieben[18]. Jedoch kann die zuständige Behörde den GR im Außenbereich aufheben[19]. Zudem dürfen die Länder abweichende Regelungen erlassen[20] und „kleine Gewässer von wasserwirtschaftlich untergeordneter Bedeutung […] von den Bestimmungen ausnehmen“[21] (siehe Kapitel 2.3.). Des Weiteren ist relevant, dass nach § 38 Abs. 4 S. 2 Nr. 3 WHG zwar der Umgang mit wassergefährdenden Stoffen in den GR allgemein verboten ist, Pflanzenschutz- und Düngemittel von diesem allgemeinen Verbot jedoch ausgenommen werden, es sei denn, dass die Länder abweichende Regelungen festlegen (siehe Kapitel 2.3.) oder dass die Anwendungsbestimmungen der spezifischen Produkte Abstände zu Gewässern vorschreiben (siehe Kapitel 2.4.). So dürfen theoretisch etwa Totalherbizide wie Roundup® UltraMax, für welche laut Anwendungsbestimmung keine Abstandsauflagen zu Gewässern vorgeschrieben sind, direkt an Gewässern appliziert werden. In diesem Beispiel kann eine Anwendung ohne GR laut Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) (2010) im schlimmsten Fall zu Konzentrationen führen, bei denen adverse Effekte auf Anurenlarven bereits im Labor nachgewiesen wurden (LAJMANOVICH *et al.* 2011, S. 681 ff.). Sollte umgekehrt aber in den GR keine landwirtschaftliche Nutzung stattfinden und Applikationen nur auf der bewirtschafteten Fläche stattfinden, sollten Stoffe wohl meist nicht direkt im GR ausgebracht werden.

Des Weiteren wurde die WRRL, die europäische Richtlinie über Umweltqualitätsnormen im Bereich der Wasserpolitik[22], die europäische Richtlinie zur Festlegung technischer Spezifikationen für die chemische Analyse und die Überwachung des Gewässerzustands[23] und die Entscheidung der europäischen Kommission zur Festlegung der Werte für die Einstufungen des Überwachungssystems des jeweiligen Mitgliedstaats als Ergebnis der

[17] § 38 Abs. 2 WHG.
[18] § 38 Abs. 3 WHG.
[19] § 38 Abs. 3 Satz 1 WHG.
[20] § 38 Abs. 3 Satz 2 WHG.
[21] § 2 Abs. 2 WHG.
[22] Richtlinie 2008/105/EG des Europäischen Parlaments und des Rates vom 16.12.2008 über Umweltqualitätsnormen im Bereich der Wasserpolitik und zur Änderung und anschließenden Aufhebung der Richtlinien des Rates 82/176/EWG, 83/513/EWG, 84/156/EWG, 84/491/EWG und 86/280/EWG sowie zur Änderung der Richtlinie 2000/60/EG (Abl. EG L 384, S. 84).
[23] Richtlinie 2009/90/EG der Kommission vom 31.07.2009 zur Festlegung technischer Spezifikationen für die chemische Analyse und die Überwachung des Gewässerzustands gemäß der Richtlinie 2000/60/EG des Europäischen Parlaments und des Rates (ABl. EU L 201, S. 36).

Interkalibrierung[24] in der OGeV[25] in deutsches Recht umgesetzt. Diese dient „dem Schutz der Oberflächengewässer und der wirtschaftlichen Analyse der Nutzungen ihres Wassers“[26]. Sie bezieht sich auch explizit auf Biozide und Pflanzenschutzmittelwirkstoffe[27], das bundesweite Monitoring von Gewässern schließt jedoch Kleingewässer < 10 km² Einzugsgebiet nicht ein[28] (siehe auch weiter unten).

Die Nitrat-Richtlinie wurde durch die DüV[29] in Bundesrecht umgesetzt. Beim Ausbringen von „Düngemitteln, Bodenhilfsstoffen, Kultursubstraten und Pflanzenhilfsstoffen mit wesentlichen Nährstoffgehalten an Stickstoff oder Phosphat“ ist ein Mindestabstand von drei Metern zur Böschungsoberkante vorgeschrieben[30]. Abweichend beträgt der Abstand nur 1 m, wenn Geräte verwendet werden, „bei denen die Streubreite der Arbeitsbreite entspricht oder die über eine Grenzstreueinrichtung verfügen“[31]. Zudem ist dafür zu sorgen, dass „kein Abschwemmen in oberirdische Gewässer erfolgt.“ Besondere Vorschriften zum Ausbringen von Düngemitteln gelten zudem auf stark zu einem Gewässer hin geneigten Flächen („Hangneigung von durchschnittlich mehr als 10 vom Hundert“), etwa dass Düngemittel hier zwischen drei und zehn Metern Abstand nur direkt in den Boden eingebracht werden dürfen[32]. Jedoch gelten diese Vorschriften der DüV nicht für Gewässer, welche von den Ländern nach § 2 Abs. 2 WHG von dessen Anwendung (und der des jeweiligen Wassergesetzes des Landes) ausgenommen sind[33].

Die Pflanzenschutz-Rahmenrichtlinie wurde im PflSchG[34] in Bundesrecht umgesetzt. Grundsätzlich ist vorgeschrieben, dass PSM „nicht in oder unmittelbar an oberirdischen Gewässern“ angewendet werden dürfen[35]. Jedoch haben auch hier die Bundesländer die Befugnis, eigene Vorschriften zur Anwendung von PSM an oberirdischen Gewässern zu

[24] Entscheidung 2008/915/EG der Kommission vom 30.10.2008 zur Festlegung der Werte für die Einstufungen des Überwachungssystems des jeweiligen Mitgliedstaats als Ergebnis der Interkalibrierung gemäß der Richtlinie 2000/60/EG des Europäischen Parlaments und des Rates (ABl. L 332, S. 20).
[25] Oberflächengewässerverordnung vom 20.07.011 (BGBl. I, S. 1429).
[26] § 1 OGeV.
[27] Anlage 2, Abs. 1 OGeV.
[28] NAP, S. 13.
[29] Düngeverordnung in der Fassung der Bekanntmachung vom 27. 02.2007 (BGBl. I S. 221), zuletzt geändert durch Art. 5 Abs. 36 des Gesetzes vom 24.02.2012 (BGBl. I S. 212).
[30] § 3 Abs. 6 DüV.
[31] § 3 Abs. 6 DüV.
[32] § 3 Abs. 7 DüV.
[33] § 3 Abs. 8 DüV.
[34] Pflanzenschutzgesetz vom 06.02.2012 (BGBl. I S. 148, 1281), zuletzt geändert durch Art. 4 Abs. 87 des Gesetzes vom 07.08.2013 (BGBl. I S. 3154).
[35] § 12 Abs. 2 PflSchG.

erlassen[36]. PSM dürfen nicht „auf befestigten Freilandflächen und nicht auf sonstigen Freilandflächen, die weder landwirtschaftlich noch forstwirtschaftlich oder gärtnerisch genutzt werden“ und nicht „an oder in unmittelbarer Nähe zu Oberflächengewässern“ angewendet werden[37], jedoch kann auch die zuständige Landesbehörde Ausnahmen für die Anwendung zugelassener PSM genehmigen, wenn „der angestrebte Zweck vordringlich ist und mit zumutbarem Aufwand auf andere Art nicht erzielt werden kann und überwiegende öffentliche Interessen, insbesondere des Schutzes der Gesundheit von Mensch und Tier oder des Naturhaushaltes, nicht entgegenstehen“[38]. § 22 PflSchG fasst die diesbezüglichen Befugnisse und Pflichten der Länder zusammen. Auf Antrag kann die jeweilige Landesbehörde selbst eine Ausbringung von PSM mit Luftfahrzeugen, was nach § 18 Abs. 1 PflSchG grundsätzlich verboten ist, genehmigen, wenn Schadorganismen im Weinbau in Steillagen bzw. im Kronenbereich von Bäumen bekämpft werden sollen[39] und bereits im Zulassungsverfahren des jeweiligen PSM eine eventuelle Ausbringung durch Luftfahrzeuge durch das BVL festgelegt wurde[40].Wichtig ist auch die Befugnis des BVL, spezifische Anwendungsbestimmungen in der Zulassung für die jeweiligen Produkte festzulegen, einschließlich solcher über „den bei sachgerechter und bestimmungsgemäßer Anwendung zum Schutz von Gewässern erforderlichen Abstand und Maßnahmen bei der Anwendung“[41]. Mit dieser Problematik befasst sich das Kapitel 2.4.

§ 4 PflSchG schreibt vor, dass die Bundesregierung einen Aktionsplan zur nachhaltigen Anwendung von PSM im Sinne des Art. 4 Abs. 1 der Pflanzenschutz-Rahmenrichtlinie beschließen soll. Der nationale Aktionsplan zur nachhaltigen Anwendung von Pflanzenschutzmitteln (NAP)[42] wurde am 10. April 2013 von der Bundesregierung beschlossen. Dieser sagt aus, dass in der Agrarlandschaft „die für den Naturhaushalt unbedenklichen Konzentrationen von Pflanzenschutzmittelwirkstoffen in Kleingewässern überschritten werden und ein guter chemischer und ökologischer Zustand oftmals noch nicht vorliegt“[43]. Über die tatsächliche Belastung von Kleingewässern in der Agrarlandschaft ist

[36] § 22 Abs. 1 PflSchG.
[37] § 12 Abs. 2 PflSchG.
[38] § 12 Abs. 2 PflSchG.
[39] § 18 Abs. 2 PflSchG.
[40] § 18 Abs. 3 PflSchG.
[41] § 36 Abs. 1 PflSchG.
[42] http://www.nap-pflanzenschutz.de/fileadmin/SITE_MASTER/content/Dokumente/Grundlagen/NAP_2013/NAP_2013.pdf.
[43] NAP, S. 13.

bisher aber wenig bekannt[44]. Ursachen hierfür sind vielfältig, aber ein behördliches Monitoring gibt es derzeit aus Kostengründen nicht[45]. Ziele des NAP diesbezüglich sind deshalb (1) „die Ermittlung des Belastungszustandes von Kleingewässern der Agrarlandschaft“[46], und (2) dass bis 2015 die Umweltqualitätsnormen für prioritäre Pflanzenschutzmittelwirkstoffe und relevante Metabolite gemäß OGeV[47] in Kleingewässern in der Agrarlandschaft eingehalten werden. Sollten keine Umweltqualitätsnormen für Stoffe vorliegen, sollen bis 2023 die im Zulassungsverfahren abgeleiteten maximal tolerierbaren Konzentrationen (RAK, Regulatorisch Akzeptable Konzentration) für Pflanzenschutzmittelwirkstoffe und relevante Metabolite in 99% der Proben eines Jahres unterschritten werden[48]. Maßnahmen hierzu sind v.a. die Erarbeitung von „Hot-Spot-Managementkonzepten“, also die Identifizierung „zeitlich und räumlich definierte(r) Aktionsfelder mit erhöhten Risiken (Hot-Spots), die mit der Anwendung von Pflanzenschutzmitteln in Verbindung stehen“ und die Erarbeitung gezielter und angepasster „Maßnahmen zur Verbesserung der Situation im Hinblick auf den Gewässerschutz“[49].

Für den Amphibienschutz relevant ist folgende Aussage: „Die Länder unterstützen im Rahmen von Agrar-Umweltprogrammen die Schaffung dauerhaft bewachsener Gewässerrandstreifen von mindestens 5 m Breite <u>an allen Oberflächengewässern</u>, insbesondere in Trinkwasserschutzgebieten, Naturschutzgebieten und in durch Hot-Spot-Analysen identifizierten sensiblen Gebieten“[50]. Dass dies derzeit noch nicht der Fall ist, wird in der vorliegenden Arbeit genauer erörtert.

2.3. Umsetzung durch landesrechtliche Regelungen

Wie bereits in Kapitel 2.2. erwähnt, dürfen die Länder vom WHG abweichende Regelungen erlassen[51] und „kleine Gewässer von wasserwirtschaftlich untergeordneter Bedeutung […] von den Bestimmungen ausnehmen“[52]. Auch gelten die Vorschriften der DüV nicht für

[44] NAP, S. 56.
[45] NAP, S. 13.
[46] NAP, S. 54.
[47] Anlagen 5 und 7 OGeV.
[48] NAP, S. 33.
[49] NAP, S. 57.
[50] NAP, S. 57.
[51] § 38 Abs. 3 Satz 2 WHG.
[52] § 2 Abs. 2 WHG.

Gewässer, welche nach § 2 Abs. 2 WHG von dessen Anwendung ausgenommen sind[53]. Umgekehrt legen manche Bundesländer breitere GR als die bundesweit geltenden 5 m fest, jedoch ist landwirtschaftliche Nutzung und Ausbringung von Pflanzenschutz- und Düngemitteln nicht immer allgemein in den GR untersagt. Daher werden im Folgenden die jeweiligen Wassergesetze in drei Gruppen unterteilt: (1) Bundesländer, welche aus naturschutzfachlicher Sicht die Vorgaben der WRRL und des WHG in Bezug auf GR gut umgesetzt haben (d.h. bis zu 10 m Breite fordern und/oder Bewirtschaftung und Ausbringung von Pflanzenschutz- und Düngemitteln in den GR allgemein untersagen), (2) Bundesländer, in denen zwar bis zu 10 m Breite der GR vorgeschrieben sind, aber Verbote der Bewirtschaftung und Applikationen von Agrochemikalien und organischen Düngemitteln im Einzelfall von der zuständigen Behörde festgelegt werden müssen und (3) Länder, welche nur 5 m Breite der GR fordern und in denen Nutzungsbestimmungen in den GR im Einzelfall festgelegt werden müssen (Tabelle 5). Zudem werden in den unterschiedlichen Wassergesetzen verschiedene Gewässer von den Bestimmungen des WHG und des jeweiligen Wassergesetzes des Landes ausgenommen. Hier ist die jeweilige Unterteilung der Gewässer in Gewässer „erster, zweiter" und teilweise „dritter Ordnung" im Landeswassergesetz relevant. Oftmals werden die ausgenommen Gewässertypen auch im jeweiligen Abschnitt über den Geltungsbereich des Gesetzes explizit genannt. Viele dieser von den Bestimmungen ausgenommenen Gewässer werden regelmäßig von Amphibien zu Reproduktionszwecken genutzt. Diese Ausnahmen flossen auch in die Unterteilung mit ein (siehe Bremen, Tabelle 5).

[53] § 3 Abs. 8 DüV.

Tab. 5: Ranking und Einteilung der Länder in drei Gruppen bzgl. der Umsetzung der Vorgaben zu GR aus naturschutzfachlicher Sicht.

Mindestbreiten für GR in den Bundesländern, die jeweilige Aufnahme bzw. Nichtaufnahme aller Kleingewässer in die Bestimmungen des WHG und des jeweiligen Landeswassergesetzes und allgemeine Verbote zum Einsatz von Dünge- und Pflanzenschutzmitteln in den GR (unabhängig von deren Anwendungsbestimmungen)

Platz	**Bundesland**	**Mindestbreite der GR [m]**	**GR an allen Kleingewässern vorgeschrieben?**	**Einsatz von Pflanzenschutz- und Düngemitteln allgemein untersagt?**
		Hoher Umsetzungsstandard		
1.	Saarland	10	Nein	Ja [a]
2.	Baden-Württemberg	10	Nein	Nur erste 5 m
	Sachsen	10	Nein	Nur erste 5 m
4.	Berlin	5	Nein	Ja
		Mittlerer Umsetzungsstandard		
5.	Bremen	5-10	Nein [b]	Ja [c]
6.	Hessen	10	Nein	Nein
7.	Sachsen-Anhalt	5-10	Nein	Nein
	Thüringen	5-10	Nein	Nein
9.	Schleswig-Holstein	5	Nein [d]	Nur erster m
		Geringer Umsetzungsstandard		
10.	Rheinland-Pfalz	5	Nein [e]	Nein
11.	Bayern	5	Nein	Nein
	Brandenburg	5	Nein	Nein
	Hamburg	5	Nein	Nein
	Mecklenburg-Vorpommern	5	Nein	Nein
	Nordrhein-Westfalen	5	Nein	Nein
16.	Niedersachsen	5	Nein [f]	Nein

[a] In den ersten 5 m dürfen keine Pflanzenschutz- und Düngemitteln, in bis zu 10 m Abstand keine Jauche, Gülle oder PSM mit Anwendungsbeschränkungen ausgebracht werden.

[b] herabgestuft, da sämtliche „kleinen Gewässer von wasserwirtschaftlich untergeordneter Bedeutung“ von den Bestimmungen ausgenommen sind.

[c] jedoch nur in GR an natürlichen Gewässern untersagt.

[d] viele kleine Gewässer und sogar kleine Seen von den Bestimmungen zu GR ausgenommen.

[e] jedoch nur Straßengräben von den Bestimmungen ausgenommen.

[f] sogar an keinerlei Gewässern, welche laut NWG der „dritten Ordnung“ zugeteilt werden.

2.3.1. Hoher Umsetzungsstandard

Das SWG[54] schließt Fischteiche und andere künstliche Gewässer ohne wasserwirtschaftliche Bedeutung, Be- und Entwässerungsgräben von wasserwirtschaftlich untergeordneter Bedeutung und Gräben, die „nicht der Vorflut oder die der Vorflut der Grundstücke nur eines Eigentümers dienen“ von den Bestimmungen aus[55]. Gewässer „erster Ordnung“ sind die Bundeswasserstraßen, Gewässer „zweiter Ordnung“ werden in einem dem Gesetz anliegendem Verzeichnis geführt; alle übrigen gelten als „dritter Ordnung“[56]. GR von 10 m Breite müssen eingehalten werden, in deren (mindestens) ersten 5 m von der Uferlinie keinerlei Ackerbau oder Anwendung von Pflanzenschutz- und mineralischen Düngemitteln erlaubt ist und in bis zu 10 m Abstand keine Jauche, Gülle oder PSM mit Anwendungsbeschränkungen ausgebracht werden dürfen[57].

Das BaWüWG[58] unterscheidet Gewässer „erster und zweiter Ordnung“. Erstere sind die Bundeswasserstraßen sowie die in Anlage 1 des Gesetzes aufgeführten Gewässer, alle übrigen zählen als „zweiter Ordnung“[59]. Vorgaben zu GR finden sich in § 29 BaWüWG. Es wird zwar allgemein ein Schutzstreifen von 10 m ab Böschungsoberkante bzw. mittleren Hochwasserstandes vorgegeben, die Wasserbehörde kann die GR breiter (aber auch schmäler) anlegen und in den ersten 5 m der GR ist die Anwendung von Pflanzenschutz- und Düngemittel allgemein untersagt, ab 2019 auch die Nutzung als Ackerland[60], jedoch sind Gewässer von „untergeordneter wasserwirtschaftlicher Bedeutung“ von den Bestimmungen des Gesetzes ausgenommen[61]. Diese sind näher definiert als „Fischteiche, Feuerlöschteiche, Eisweiher und ähnliche kleine Wasserbecken“[62] sowie „Be- und Entwässerungsgräben“[63]. Alle diese Gewässer werden regelmäßig von verschiedenen Amphibienarten zur Reproduktion genutzt. Etwa sind Feuerlöschteiche im ländlichen Bereich wichtige Fortpflanzungsgewässer

[54] Saarländisches Wassergesetz vom 28.06.1960 (Amtsbl., S. 1994), zuletzt geändert durch das Gesetz vom 03.12.2013 (Amtsbl., S. 2).
[55] § 1 Abs. 2 SWG.
[56] § 3 Abs. 1 SWG.
[57] § 56 Abs. 3 SWG.
[58] Wassergesetz für Baden-Württemberg vom 03.12.2013 (GBl. 17, S. 389).
[59] § 4 BaWüWG.
[60] § 29 Abs. 3 BaWüWG.
[61] § 29 Abs. 1 BaWüWG.
[62] § 2 Abs. 2 BaWüWG.
[63] § 2 Abs. 3 BaWüWG.

der Geburtshelferkröte (GÜNTHER 1996, S. 195 ff.), welche in der Roten Liste von Baden-Württemberg als „stark gefährdet“ gelistet ist (Tabelle 3).

Das SächsWG [64] schließt folgende Gewässer mit wasserwirtschaftlich untergeordneter Bedeutung von den Bestimmungen aus: Gräben, die ausschließlich das Grundstück eines einzigen Eigentümers be- oder entwässern, Straßenseitengräben, Fischteiche und andere künstlich errichteten Gewässer, die nicht oder nur künstlich mit einem anderen Gewässer in Verbindung stehen und kleine Fließgewässer (bis 500 m von der Quelle bis zur Mündung)[65]. Das SächsWG unterscheidet Gewässer „erster und zweiter Ordnung“; erstere sind in Anlage 3 des Gesetzes aufgeführt, alle übrigen sind „zweiter Ordnung“[66]. Der allgemeine GR soll im Außenbereich 10 m breit sein, wobei jedoch nur in den ersten 5 m explizit die Anwendung von Pflanzenschutz- und Düngemitteln verboten ist[67]. Die zuständige Wasserbehörde kann auch hier die Breite der GR abweichend gestalten, diese jedoch nicht gänzlich aufheben[68].

Das BWG[69] unterteilt Gewässer nur in „Gewässer erster Ordnung“ (in Anlage 1 genannt, d.h. Kanäle und große Seen) und „Gewässer zweiter Ordnung“ (alle anderen Gewässer)[70]. Bestimmte Kleingewässer sind von den Bestimmungen des WHG und des BWG ausgenommen: Seitengräben von Straßen und Eisenbahnlinien, zeitweilig wasserführenden Gräben, Be- und Entwässerungsgräben und künstliche Gewässer (etwa Fischteiche)[71]. Zudem sind zwar GR an allen Gewässern erster Ordnung vorgeschrieben, jedoch nur an fließenden Gewässern zweiter Ordnung[72]. Da es keine weiteren abweichenden Änderungen gegenüber dem WHG bzgl. GR gibt, ist die bundesweit gültige GR-Breite von 5 m einzuhalten. Jedoch ist in den GR die Anwendung von Pflanzenschutz- und Düngemitteln sowie die Ackernutzung allgemein untersagt[73].

[64] Sächsisches Wassergesetz vom 12.07.2013 (GVBl., S. 503).
[65] § 1 Abs. 2 SächsWG.
[66] § 30 Abs. 1 SächsWG.
[67] § 24 Abs. 3 SächsWG.
[68] § 24 Abs. 4 SächsWG.
[69] Berliner Wassergesetz vom 17.06.2005 (GVBl., S. 48), zuletzt geändert durch das Gesetz vom 06.06.2008 (GVBl., S. 139).
[70] § 2 BWG.
[71] § 1 Abs. 2 BWG.
[72] § 40a Abs. 1 BWG.
[73] § 40a Abs. 2 BWG.

2.3.2. Mittlerer Umsetzungsstandard

Nach dem BremWG[74] sind alle kleinen Gewässer von wasserwirtschaftlich untergeordneter Bedeutung von den Bestimmungen ausgenommen[75]. Dies dürfte einen Großteil – wenn nicht den Hauptteil – der Amphibienlaichgewässer Bremens beinhalten, was die Herabstufung Bremens in die zweite Ländergruppe erklärt. Als „Gewässer erster Ordnung" werden die Bundeswasserstraßen, Hafengewässer sowie weitere Flüsse, die in § 3 Abs. 1 BremWG klassifiziert sind. Als „Gewässer zweiter Ordnung" gelten alle anderen Gewässer, mit Ausnahme von „Gräben, die nicht dazu dienen, die Grundstücke mehrerer Eigentümer zu bewässern oder zu entwässern", welche als Gewässer „dritter Ordnung" definiert sind[76]. Abweichend von § 38 WHG müssen im Außenbereich 10 m GR eingehalten werden[77], um Be- und Entwässerungsgräben 5 m [78](darunter fallen auch die im BremWG als „Gewässer dritter Ordnung" definierten Gräben). Jedoch ist die allgemeine Anwendung von Pflanzenschutz- und Düngemitteln nur in den GR von natürlichen Gewässern verboten[79], was nach § 3 Abs. 3 BremWG definierte „künstliche Gewässer" (d.h. in einem künstlichen Bett errichteten) nicht miteinschließt, welche jedoch sehr wohl von Amphibien als Laichgewässer genutzt werden können.

Das HWG[80] schließt Straßengräben, alle Be- und Entwässerungsgräben sowie Fischteiche und andere künstliche Gewässer ohne wasserwirtschaftliche Bedeutung von den Bestimmungen aus[81]. Jedoch gilt das Gesetz auch für das aus Niederschlägen stammende Wasser, welches wild abfließt[82]. Hier kann diskutiert werden, ob temporär überflutete Felder und Wiesen, welche oftmals von verschiedenen Amphibienarten zu Reproduktionszwecken genutzt werden (z.B. BATTAGLIN *et al.* 2009, S. 281 ff.) daher unter die Bestimmungen des WHG und des HWG fallen. Die nach § 2 HWG in „erster und zweiter Ordnung" unterteilten Gewässer sind in den Anlagen 1-2 des Gesetzes aufgezählt (hierzu zählen nur Fließgewässer). Alle anderen

[74] Bremisches Wassergesetz vom 12.04.2011 (GBl., S. 262), zuletzt geändert durch das Gesetz vom 23.04.2013 (GBl., S. 131).
[75] § 2 BremWG.
[76] § 3 Abs. 1 BremWG.
[77] § 21 Abs. 1 BremWG.
[78] § 21 Abs. 2 BremWG.
[79] § 21 Abs. 3 BremWG.
[80] Hessisches Wassergesetz vom 14.12.2010 (GVBl., S. 548), zuletzt geändert durch Art. 62 des Gesetzes (GVBl., S. 622).
[81] § 1 Abs. 2 HWG.
[82] § 1 Abs. 1 HWG.

Gewässer gelten als „dritter Ordnung“[83]. Die Breite der GR im Außenbereich wird auf 10 m festgesetzt und kann wiederum durch Rechtsverordnung abweichend geregelt werden[84].

Das WG LSA[85] schließt alle Gräben von den Bestimmungen aus, die nicht dazu dienen, die Grundstücke anderer Eigentümer zu bewässern sowie Fischteiche und andere künstliche Gewässer, die nicht oder nur künstlich mit einem anderen Gewässer in Verbindung stehen[86]. Gewässer „erster Ordnung“ sind die Bundeswasserstraßen sowie in Anlage 1 des Gesetzes aufgeführte Gewässer (inklusive einiger Tagebaurestseen)[87]. Alle übrigen Gewässer sind „zweiter Ordnung“[88]. Die Breite der GR beträgt 10 m für Gewässer „erster Ordnung“ und 5 m für Gewässer “zweiter Ordnung“[89]. Ein explizites Verbot der Verwendung von Pflanzenschutz- und Düngemittel muss durch die Wasserbehörde im Einzelfall erfolgen, falls dies nach § 38 Abs. 1 WHG erforderlich ist[90].

Das ThürWG[91] schließt Straßengräben, zeitweilig wasserführende Gräben, Be- und Entwässerungsgräben sowie Fischteiche und andere künstliche Gewässer, welche nicht oder nur künstlich mit einem anderen Gewässer in Verbindung stehen, von den Bestimmungen aus[92]. Gewässer „erster Ordnung“ sind in Anlage 1 des Gesetzes genannt, alle übrigen gelten als „zweiter Ordnung“[93]. Der GR muss bei Gewässern „erster Ordnung“ 10 m, bei Gewässern „zweiter Ordnung“ 5 m betragen[94], PSM dürfen hierin aber nach ihren Anwendungsbestimmungen und Düngemittel nach den Bestimmungen der DüV ausgebracht werden[95].

Das WasG SH[96] schließt Gräben und kleine Wasseransammlungen, die nicht oder nur der Vorflut eines Eigentümers dienen sowie Fischteiche und andere künstliche Gewässer, die nicht oder nur künstlich mit einem anderen Gewässer verbunden sind, von den Bestimmungen aus[97]. Gewässer „erster Ordnung“ sind wiederum die Bundeswasserstraßen sowie die in

[83] § 2 HWG.
[84] § 23 Abs. 1 HWG.
[85] Wassergesetz für das Land Sachsen-Anhalt vom 16.03.2011 (GVBl., S. 492).
[86] § 1 Abs. 2 WG LSA.
[87] § 4 WG LSA.
[88] § 5 WG LSA.
[89] § 50 Abs. 1 WG LSA.
[90] § 50 Abs. 4 WG LSA.
[91] Thüringer Wassergesetz vom 18.08.2009 (GVBl., S. 648).
[92] § 1 Abs. 2 ThürWG.
[93] § 3 Thür WG.
[94] § 78 Abs. 2 ThürWG.
[95] § 78 Abs. 3 ThürWG.
[96] Wassergesetz des Landes Schleswig-Holstein vom 11.02.2011 (GVBl., S. 91).
[97] § 1 Abs. 2 WasG SH.

Anlage 2 des Gesetzes aufgeführten Gewässer, „zweiter Ordnung“ alle übrigen Gewässer[98]. Es besteht keine GR-Pflicht an folgenden Gewässern „zweiter Ordnung“: die ein Gebiet von weniger als 20 ha entwässern, die keine besondere Bedeutung für die Vorflut besitzen, die überwiegend der Entwässerung von Verkehrsflächen dienen (etwa Regenrückhaltebecken)[99] sowie allgemein an Seen mit einer Fläche von weniger als 1 ha[100]. Zudem ist in den 5 m breiten GR nur im ersten Meter landseits das Pflügen von Ackerland und die Anwendung von Pflanzenschutz- und Düngemitteln verboten[101]. Die oberste Wasserbehörde kann jedoch auch in Schleswig-Holstein im Einzelfall die Breite abweichend festlegen, eine Bewirtschaftung und Anwendung von Pflanzenschutz- und Düngemitteln gänzlich untersagen[102].

2.3.3. Niedriger Umsetzungsstandard

Das LWG für Rheinland-Pfalz[103] schließt nur Straßenseitengräben von den Bestimmungen aus[104]. Gewässer „erster Ordnung“ sind in Anlage 1 des Gesetzes aufgeführt, „zweiter Ordnung“ in einem Verzeichnis der obersten Wasserbehörde, die übrigen Gewässer gelten als „dritter Ordnung“[105]. Abweichungen bzgl. der Breite werden nicht aufgeführt, so dass die bundesweite Breite von 5 m gilt. Auch hier kann die zuständige Behörde (bei Gewässern „erster und zweiter Ordnung“ die obere, bei Gewässern „dritter Ordnung“ die untere Wasserbehörde[106]) für bestimmte Gewässer GR und deren Breite sowie Auflagen darin per Rechtsverordnung festlegen[107]. Auch Regelungen über die Anwendung von Dünge- und Pflanzenschutzmitteln müssen im Einzelfall in dieser Rechtsverordnung geregelt werden[108].

Im BayWaG[109] werden als „Gewässer erster Ordnung“ die Bundeswasserstraßen sowie die in Anlage 1 des Gesetzes aufgeführten Gewässer definiert (große Flüsse und Seen)[110].

98 § 3 Abs. 1 WasG SH.
99 § 40 Abs. 2 WasG SH.
100 § 38a Abs. 1 WasG SH.
101 § 38a Abs. 2 WasG SH.
102 § 38a Abs. 3 WasG SH.
103 Wassergesetz für das Land Rheinland-Pfalz vom 22.01.2004 (GVBl., S. 54), zuletzt geändert durch Art. 2 des Gesetzes vom 23.11.2011 (GVBl., S. 402).
104 § 1 LWG.
105 § 3 Abs. 2 LWG.
106 § 15a Abs. 4 LWG.
107 § 15a Abs. 1 LWG.
108 § 15a Abs. 2 LWG.
109 Bayerisches Wassergesetz vom 25.02.2010 (GVBl., S. 66).
110 Art. 2 Abs. 1 Nr. 1 BayWG.

„Gewässer zweiter Ordnung“ werden in einem Gewässerverzeichnis geführt[111], welche „wasserwirtschaftlich, insbesondere wegen ihrer Wasser-, Geschiebe-, Schwebstoff- oder Eisführung, wegen ihrer ökologischen Funktionen oder wegen ihrer Nutzbarkeit von größerer Bedeutung sind“, insbesondere typische Wildbäche[112]. „Gewässer dritter Ordnung“ sind alle übrigen Gewässer[113]. Von den Bestimmungen des Gesetzes ausgenommen sind neben allen Be- und Entwässerungsgräben, kleine Teiche und Weiher, welche nicht oder nur künstlich mit einem anderen Gewässer in Verbindung stehen[114]. An den (natürlichen) Gewässern dritter Ordnung können GR durch „Anordnung für den Einzelfall oder durch Rechtsverordnung von der Kreisverwaltungsbehörde im Einvernehmen mit den Trägern der Gewässerunterhaltung“ festgesetzt werden „wenn ohne eine Festsetzung […] die Erreichung der Bewirtschaftungsziele nach Maßgabe der §§ 27 bis 31 WHG gefährdet ist“[115]. Dies bedeutet besonders, dass „eine Verschlechterung ihres ökologischen und ihres chemischen Zustands vermieden wird“[116] und „ein guter ökologischer und ein guter chemischer Zustand erhalten oder erreicht werden“[117]. Folglich werden für Kleingewässer nur dann GR vorgeschrieben, wenn explizit nachgewiesen wird, dass ohne GR z.B. die Fortpflanzung lokaler Amphibienpopulationen negativ beeinflusst wird. Ansonsten gelten bzgl. GR keine weiteren für den Amphibienschutz relevanten abweichenden Änderungen gegenüber dem WHG, so dass auch eine allgemeine GR-Breite von 5 m einzuhalten ist.

Das BbgWG[118] sieht in „Gewässern erster Ordnung“ Bundeswasserstraßen und Gewässer, welche von dem für Wasserwirtschaft zuständigen Mitglied der Landesregierung festgelegt werden können; alle übrigen Gewässer sind „zweiter Ordnung“[119]. Bestimmte Gräben, Fischteiche und andere künstliche Gewässer ohne wasserwirtschaftliche Bedeutung werden von den Bestimmungen des Gesetzes ausgenommen[120]. Abweichend von den Regelungen für GR nach WHG kann das für die Wasserwirtschaft zuständige Mitglied der Landesregierung „den örtlichen Verhältnissen entsprechend die Breite von Gewässerrandstreifen sowie das Verhalten im Gewässerrandstreifen für Gewässer oder Gewässerabschnitte durch

[111] Art. 2 Abs. 1 Nr. 2 BayWG.
[112] Art. 3 Abs. 1 BayWG.
[113] Art. 2 Abs. 1 Nr. 3 BayWG.
[114] Art. 1 Abs. 1 BayWG.
[115] Art. 21 Abs. 2 BayWG.
[116] § 27 Abs. 1 Nr. 1 WHG.
[117] § 27 Abs. 1 Nr. 2 WHG.
[118] Brandenburgisches Wassergesetz vom 02.03.2012 (GVBl., S. 12).
[119] § 3 BbgWG.
[120] § 1 Abs. 4 BbgWG.

Rechtsverordnung regeln, soweit es […] zur Vermeidung oder Verminderung von Schadstoffeinträgen erforderlich ist“[121].

Das HWaG[122] schließt Fischteiche, Straßengräben und Gräben, die „nicht der Vorflut oder die der Vorflut von Grundstücken nur eines Eigentümers dienen“, vom Geltungsbereich aus[123]. „Gewässer erster Ordnung“ werden in der Anlage aufgeführt (große Flüsse und Seen); alle übrigen Gewässer gelten als „zweiter Ordnung“[124]. Der Senat kann durch Rechtsverordnung die Breite von GR festsetzen, aber auch aufheben[125].

Das LWG für Mecklenburg-Vorpommern [126] schließt Fischteiche und andere künstliche Gewässer ohne wasserwirtschaftliche Bedeutung von seinen Bestimmungen aus, ebenso Gräben und „kleinere Wasseransammlungen“. Für zeitweilig wasserführende Kleinstgewässer sind keinerlei Abstände und keine Anwendungsbeschränkungen für Dünge- und Pflanzenschutzmitteleinsätze zu beachten [127]. Solche flachen Temporärgewässer in der Kulturlandschaft werden jedoch häufig etwa von der Kreuzkröte zu Reproduktionszwecken genutzt (GÜNTHER 1996, S. 302 ff.), welche in der Roten Liste von Mecklenburg-Vorpommern als „stark gefährdet“ gelistet ist (Tabelle 3). Gewässer werden nur in „erste und zweite Ordnung“ unterteilt, wobei erstere Bundeswasserstraßen und die in Anlage 1 des Gesetzes aufgeführten Fließgewässer beinhalten, alle übrigen als „zweiter Ordnung“ gelten[128]. Gesonderte Bestimmungen zu GR finden sich nicht, so dass die bundesweite Breite von 5 m gilt.

Das LWG Nordrhein-Westfalen [129] nimmt Entwässerungsgräben, welche nicht „der Vorflut der Grundstücke anderer Eigentümer dienen“ von den Bestimmungen aus[130], sowie nach § 3 Abs. 1 LWG alle Straßenseitengräben. Gewässer „erster und zweiter Ordnung“ werden in Anlage 2 des Gesetzes aufgeführt; alle übrigen Gewässer werden „sonstige Gewässer“

[121] § 84 Abs. 2 BbgWG.
[122] Hamburgisches Wassergesetz vom 29.03.2005 (GVBl., S. 97), zuletzt geändert durch Art. 12 des Gesetzes vom 04.12.2012 (GVBl., S. 510).
[123] § 1 Abs. 2 HWaG.
[124] §2 HWaG.
[125] §26a HWaG.
[126] Wassergesetz des Landes Mecklenburg-Vorpommern vom 30.11.1992 (GVBl., S. 669), zuletzt geändert durch Art. 4 des Gesetzes vom 04.07.2011 (GVBl., S. 759).
[127] § 1 Abs. 2 LWaG.
[128] § 48 Abs. 1 LWaG.
[129] Wassergesetz für das Land Nordrhein-Westfalen vom 25.06.1995 (GV., S. 926), zuletzt geändert durch Art. 3 des Gesetzes vom 16.03.2010 (GV., S. 185).
[130] § 1 Abs. 1 LWG.

genannt[131]. Die Breite der GR im Außenbereich beträgt 5 m[132] und wie auch in den übrigen Bundesländern kann die zuständige Behörde die Breite im Einzelfall abweichend festlegen oder GR aufheben[133]. Für den Amphibienschutz relevant ist auch, dass PSM, deren Anwendungsbestimmungen es erlauben (siehe Kapitel 2.4.), in den GR explizit angewendet werden dürfen[134].

Das NWG[135] schließt Gräben, Fischteiche sowie andere künstliche Gewässer, die ohne wasserwirtschaftlichen Zweck errichtet wurden, von den Bestimmungen aus[136]. Gewässer „erster Ordnung" sind Bundeswasserstraßen sowie in Anlage 3 des Gesetzes aufgeführte Gewässer[137], „zweiter Ordnung" Gewässer von „überörtlicher Bedeutung", welche in einem speziellen Verzeichnis der Wasserbehörde gelistet werden[138], „dritter Ordnung" alle übrigen Gewässer[139]. Zur Breite der GR werden keine gesonderten Angaben getätigt, so dass die bundesweiten 5 m gelten. Jedoch besteht an sämtlichen Gewässern „dritter Ordnung" kein GR[140], was eine Vielzahl – wenn nicht den Hauptteil – der niedersächsischen Amphibienlaichgewässer beinhalten dürfte. Zudem muss ein allgemeines Anwendungsverbot von Dünge- und Pflanzenschutzmitteln von der Wasserbehörde im Einzelfall festgelegt werden[141].

2.4. Formulierungsspezifische Vorgaben nach dem Pflanzenschutzgesetz

Das PflSchG teilt dem BVL eine Vielzahl spezieller Aufgaben zu. Das BVL wirkte bei der Erarbeitung des NAP mit[142]. Die zuständigen Landesbehörden müssen jährlich dem BVL Ausnahmegenehmigungen nach § 12 Abs. 2 Satz 3 PflSchG melden[143]. Das BVL muss im elektronischen Bundesanzeiger die Aufbrauchfirsten für PSM bekannt geben, deren Zulassung

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[131] § 3 Abs. 1 LWG.
[132] § 90a Abs. 1 LWG.
[133] § 90a Abs. 4 LWG.
[134] § 90a Abs. 2 LWG.
[135] Niedersächsisches Wassergesetz vom 19.02.2010 (GVBl., S. 64), zuletzt geändert durch § 87 Abs. 3 des Gesetzes vom 03.04.2012 (GVBl., S. 46).
[136] § 1 Abs. 1 NWG.
[137] § 38 NWG.
[138] § 39 NWG.
[139] § 40 NWG.
[140] § 58 Abs. 1 NWG.
[141] § 58 Abs. 2 NWG.
[142] § 5 PflSchG.
[143] § 12 Abs. 2 PflSchG.

abgelaufen ist[144] und die Rückgabe solcher PSM anordnen[145]. Auch die Anwendung von PSM auf Flächen, die für die Allgemeinheit bestimmt sind (also keine land-, forstwirtschaftlich oder gärtnerisch genutzten Flächen), kann nur das BVL mit Einverständnis der Bundesamtes für Risikobewertung, dem Julius-Kühn-Institut und dem Umweltbundesamt genehmigen, wenn für die Anwendung ein „öffentliches Interesse“ besteht und „eine Prüfung ergibt, dass das Pflanzenschutzmittel auf Grund seiner chemischen Eigenschaften bei bestimmungsgemäßer und sachgerechter Anwendung keine schädlichen Auswirkungen auf die Allgemeinheit hat“[146]. Hierzu kann das BVL spezifische, von der Zulassung des Mittels abweichende Anwendungsbestimmungen festlegen[147]. Eine Liste der Mittel, die auch außerhalb land-, forstwirtschaftlich oder gärtnerisch genutzten Flächen angewendet werden dürfen, muss vom BVL ebenfalls im elektronischen Bundesanzeiger veröffentlicht werden[148]. Bei „Gefahr im Verzug“ kann eine Landesbehörde auf solchen Flächen auch die Anwendung anderer PSM als solcher, die „mit geringem Risiko nach Art. 47 der Verordnung (EG) Nr. 1107/2009 zugelassen“ sind genehmigen und muss das BVL darüber informieren[149]. Auch das eventuelle Ausbringen von PSM mit Luftfahrzeugen, was nach § 18 Abs. 1 PflSchG grundsätzlich verboten ist, darf vom BVL für ein PSM im Zulassungsverfahren festgelegt werden, wenn, im Benehmen mit dem Bundesamt für Risikobewertung, dem Julius-Kühn Institut und dem Umweltbundesamt, „eine Prüfung ergibt, dass das Pflanzenschutzmittel auf Grund seiner Eigenschaften bei bestimmungsgemäßer und sachgerechter Anwendung auch bei der Anwendung mit Luftfahrzeugen keine schädlichen Auswirkungen auf die Gesundheit von Mensch und Tier oder auf Grundwasser und keine sonstigen nicht vertretbaren Auswirkungen auf den Naturhaushalt hat“[150]. Eine Liste der PSM, deren Ausbringung durch Luftfahrzeuge die zuständige Landesbehörde auf Antrag genehmigen darf, muss das BVL im elektronischen Bundesanzeiger veröffentlichen[151]. Auch darf das BVL die Ausbringung nicht zugelassener PSM zu Versuchszwecken oder Versuche mit zugelassenen PSM auf nicht zugelassenen Flächen genehmigen, „soweit durch den Versuch oder das Versuchsprogramm keine schädlichen Auswirkungen auf die Gesundheit von Mensch und Tier oder sonstige nicht

[144] § 12 Abs. 5 PflSchG.
[145] § 27 Abs. 2 PflSchG.
[146] § 17 Abs. 2 PflSchG.
[147] § 17 Abs. 3 PflSchG.
[148] § 17 Abs. 4 PflSchG.
[149] § 17 Abs. 6 PflSchG.
[150] § 18 Abs. 4 PflSchG.
[151] § 18 Abs. 6 PflSchG.

vertretbare Auswirkungen auf den Naturhaushalt zu erwarten sind“[152]. Das BVL hat die Gelegenheit zur Stellungnahme bevor durch die zuständigen Landesbehörden Ausnahmegenehmigungen nach § 22 Abs. 2 PflSchG erteilt werden[153]. Zudem muss jeder, der gewerblich mit PSM handelt, sich zuvor beim BVL anmelden[154].

Zusammenfassend kann gesagt werden, dass der Gesetzgeber mit dem PflSchG dem BVL eine entscheidende Rolle in der Bewertung der Umweltverträglichkeit von PSM zugeteilt hat, etwa bei der Erteilung von Ausnahmegenehmigungen, wobei die wichtigste Aufgabe des BVL nach PflSchG diejenige ist, PSM in Deutschland direkt zuzulassen und deren Anwendungsvorschriften festzulegen[155] (bis auf wenige Ausnahmen, welche in § 28 Abs. 2 PflSchG geregelt sind).

3. Rechtliche Bewertung von Beeinträchtigungen durch den Einsatz von Pflanzenschutz- und Düngemittel aus Sicht des Artenschutzrechtes

3.1. Verbotstatbestände nach § 44 BNatSchG

Wie bereits in der Einleitung (Kapitel 1.2.) dargestellt, sind viele der einheimischen Amphibienarten und alle, welche als „Offenlandarten“ ein erhöhtes Risiko gegenüber Pestizidexposition besitzen (Tabelle 4), in Anhang IV der FFH-Richtlinie gelistet, für die ein sogenanntes „strenges Schutzsystem“ vorgesehen ist. Dieser Schutz umfasst insbesondere die in Art. 12 Abs. 1 lit. a) bis d) der FFH-Richtlinie dargestellten Verbote, welche in Deutschland in § 44 Abs. 1 Nr. 1 bis 4 BNatSchG abgebildet sind (Zugriffsverbote). Diese beinhalten u. a. das Verbot des Tötens von Exemplaren (d.h. Schutz auf der Individualebene) dieser Arten sowie der Beschädigung und Vernichtung von Fortpflanzungs- oder Ruhestätten.

Gegen einen strengen Artenschutz direkt auf der landwirtschaftlich genutzten Fläche spricht, dass der strenge Artenschutz teilweise nach europarechtlichen Grundlagen [156] aufgehoben wird, wenn Individuen durch die Bewirtschaftung getötet werden, durch die Bewirtschaftung

[152] § 20 Abs. 2 PflSchG.
[153] § 22 Abs. 4 PflSchG.
[154] § 24 Abs. 2 PflSchG.
[155] Abschnitt 6 PflSchG.
[156] Vgl. Leitfaden der Kommission: Guidance document on the strict protection of animal species of Community interest under the Habitats Directive 92/43/EEC, Final version, February 2007, S. 11.

aber ihr Lebensraum überhaupt erhalten wird (etwa Tötung einzelner Feldhamster durch Mähmaschinen). Bei Offenlandarten (vgl. Tabelle 4), welche durch die Landwirtschaft frei gehaltene Flächen als Lebensraum nutzen (auch wenn diese sekundär besiedelt werden oder etwa ehemalige Auenamphibien hier persistieren) könnte so argumentiert werden. Der Leitfaden der Kommission spricht jedoch hier insbesondere von traditionellen Landwirtschaftsformen, welche vielerlei Habitate erschaffen. Intensivierung der Landwirtschaft, welche zu einem Rückgang von streng geschützten Arten führt, muss hingegen verhindert werden[157]. Tatsache ist, dass durch mechanische als auch chemische landwirtschaftliche Bearbeitungsprozesse Amphibien getötet werden können und werden. Ob dies aber einen Einfluss auf die lokale Population darstellt, ist meist nur schwer abzuschätzen, da Daten zu Populationsgrößen in der Agrarlandschaft oftmals fehlen, was wiederum hervorhebt, wie wichtig Grundlagendaten zu Populationsgrößen und –entwicklungen sind (vgl. WAGNER *et al.* 2013, S. 1; siehe Kapitel 3.3.2.).

Die nach Landesrecht zuständigen Naturschutzbehörden sind zudem ermächtigt, Ausnahmen von den Verbotstatbeständen zuzulassen[158]. Ein Ausnahmetatbestand stellt die Abwendung von landwirtschaftlichen Schäden dar[159]. Ein Schaden liegt seit der Kleinen Novelle des BNatSchG aus dem Jahre 2007 nicht nur vor, wenn es sich um einen gemeinwirtschaftlichen Schaden handelt, sondern es werden auch ernstzunehmende indivuelle Schäden erfasst[160], d.h. wenn es zu einer Beeinträchtigung oder Verschlechterung der wirtschaftlichen Grundlage einzelner Betriebe kommt[161]. Auch europarechtlich lässt Art. 16 Abs. 1 lit. b der FFH-Richtlinie Ausnahmen zur „Verhütung ernster Schäden“ etwa an Kulturen, Gewässern und sonstigem individuellem Eigentum zu[162]. Die Erheblichkeit muss im Einzelfall entschieden werden, d.h. wo die dem Einzelnen zumutbare Belastungsgrenze liegt[163].

Ein spezielles Problem stellen über landwirtschaftlichen Flächen wandernde und auf ihnen rastende bzw. nahrungssuchende Amphibien dar, für welche die Fläche nicht unbedingt ein lange genutztes Habitat, jedoch zumindest ein Teilhabitat darstellt. Diese können durch Pestizidapplikationen stark geschädigt werden (bis zu 100% Mortalität bei empfohlenen

[157] Leitfaden der Kommission: Guidance document on the strict protection of animal species of Community interest under the Habitats Directive 92/43/EEC, Final version, February 2007, S. 31.
[158] § 45 Abs. 7 BNatSchG.
[159] § 45 Abs. 7 S. 1 Nr. 1 BNatSchG.
[160] SCHÜTTE/GERBIG, in: SCHLACKE,GK-BNatSchG, § 45 Rdn. 21, S. 626.
[161] KRATSCH, in: SCHUMACHER/FISCHER-HÜFTLE, Kom BNatSchG, § 45 Rdn. 32, S. 771.
[162] FRENZ/LAU, in: FRENZ/MÜGGENBORG, Kom BNatSchG, § 45 Rdn. 27, S. 900.
[163] SCHÜTTE/GERBIG, in: SCHLACKE,GK-BNatSchG, § 45 Rdn. 22, S. 626.

Applikationsmengen: BRÜHL *et al.* 2013, S. 1135 ff.). Dies kann fast alle einheimischen Amphibien betreffen (vgl. Tabelle 4), also auch solche, welche den Großteil des Jahres in entfernten Gebieten verbringen (etwa im Wald). Primär besteht hier wohl nur die Möglichkeit einer freiwilligen Zusammenarbeit von Feldherpetologen, Naturschutzbehörden und Landwirten. Etwa können Absprachen getroffen werden, dass Applikationen von Agrochemikalien nicht zu den Hauptwanderzeiten der im Gebiet vorkommenden Amphibienarten stattfinden (HENLE *et al.* 2008, S. 67).

Umweltrelevante bzw. „legale" Konzentrationen von in Deutschland zugelassenen PSM in angrenzenden Habitaten (Fortpflanzungsgewässern oder Landlebensräume) leiten sich daraus ab, dass ein sogenanntes „Toxicity Exposure Ratio (TER)" in der Zulassung nicht unterschritten wird[164]. D.h. dass die im Labor beobachteten Konzentrationen, welche einen Effekt auf Labortestorganismen besitzen, durch die maximal geschätzte Konzentration in der Nahrung bzw. im Falle aquatischer Organismen des Wassers geteilt wird und ein Produkt als unbedenklich betrachtet wird, wenn z.B. im Falle der akuttoxischen Wirkung (also produktbedingte Mortalität) dieser über 10 liegt[165]. Als Effektkonzentration wird hier aber der LC50-Wert benutzt, d.h. die Dosis, welche die Hälfte der Labortiere getötet hat. Folglich wird ein gewisser Anteil produktbezogener Mortalität bei Wildtieren toleriert, was streng genommen gegen den individuellen Schutz von Anhang IV-Arten steht. Jedoch könnte auch hier mir einem ernsten wirtschaftlichen Schaden argumentiert werden.

Zudem gibt es zwei weitere, grundlegende Probleme bei der Bewertung toxischer Effekte auf Amphibien: (1) Weder in der EU noch den USA sind Amphibien Standardtestorganismen, sondern die Effektkonzentrationen werden für adulte Tiere von den Daten von Säugern und Vögeln (z.B. akute Toxizität über die Nahrung) bzw. für aquatische Larven von den Daten von Fischen und Invertebraten (z.B. Daphnien) abgeleitet (RELYEA 2011, S. 267 ff.). Dies ist jedoch aus wissenschaftlicher Sicht aus vielerlei Gründen nicht nachvollziehbar, da sich etwa Parameter zur oralen Aufnahme von Stoffen über die Nahrung bei Vögeln und Amphibien stark unterscheiden und bei Amphibien die Aufnahme über die Haut (dermale Absorption) viel relevanter ist als bei Säugern und Vögeln (QUARANTA *et al.* 2009, S. 1 ff.). Folglich könnten „legale" Umweltkonzentrationen für manche Wildtiere (inklusive streng geschützter Amphibien) viel toxischer sein als aus Laborversuchen bekannt. (2) Im Freiland kommt es zu vielen Wechselwirkungen zwischen abiotischen und biotischen Zusatzfaktoren und der

[164] siehe z.B. http://www.oecd.org/chemicalsafety/pesticides-biocides/1944146.pdf.
[165] siehe z.B. http://ec.europa.eu/food/plant/pesticides/approval_active_substances/docs/wrkdoc10_en.pdf.

Toxizität von Umweltgiften (für z.B. glyphosatbasierte Herbizide zusammengefasst in WAGNER & LÖTTERS 2013a, S. 112 ff.). Zwar werden in manchen Risikobewertungen sogenannte „higher tier data“ verwendet (d.h. aus semi-realistischen Mesokosmen-Versuchen[166]), jedoch ist dies nicht die Regel und alle Faktoren aus einem komplexen Ökosystem im Freiland und ihre Auswirkungen und Wechselbeziehungen sind streng genommen nicht abbildbar (WAGNER & LÖTTERS 2013a, S. 112 ff.). Folglich besteht noch viel Forschungsbedarf bei der Frage der Auswirkungen von Pestizideinsätzen auf natürliche Amphibienpopulationen. Auch hier müssen wiederum die fehlenden Grundlagendaten tatsächlicher Habitatkontamination als auch zur Verbreitung und Bestandsdichten von Amphibien erwähnt werden (siehe Kapitel 3.3.2.).

Letztlich könnten Fortpflanzungs- oder Ruhestätten beschädigt oder vernichtet werden, wenn Konzentrationen von PSM in Gewässern adulte Amphibien davon abhalten, diese zu nutzen. Die Studien hierzu sind jedoch spärlich und kommen zu unterschiedlichen Aussagen (TAKAHASHI 2007, S. 1476 ff.; VONESH & BUCK 2007, S, 219 ff.; VONESH & KRAUS 2009, S. 379 ff.; WAGNER & LÖTTERS 2013b, S. 98 ff.). Sicher jedoch können legale Düngemitteleinträge die Eutrophierung von Laichgewässern beschleunigen, was zuerst jedoch sogar positive Effekte auf Amphibienlarven (nicht jedoch andere Taxa!) haben kann (durch erhöhtes Nahrungsangebot an etwa Algen, erhöhte Versteckmöglichkeiten); eine zu starke Eutrophierung kann jedoch zu anoxischen Verhältnissen und dadurch zum Tod der Tiere führen (siehe MANN *et al.* 2009, S. 2093 ff.).

3.2. Möglichkeiten zu verbesserten Vermeidungs- und Minimierungsmaßnahmen

3.2.1. Ausweisung von Gewässerrandstreifen um Amphibienlaichgewässer

Dem Problem, dass Amphibienlaichgewässer oftmals auf Grundlage der jeweiligen Wassergesetze von den Schutzbestimmungen gemäß WHG ausgeschlossen sind, könnte mit Hilfe nationaler (BArtSchV, BNatSchG) und europarechtlicher (FFH-Richtlinie) Vorgaben entgegengewirkt werden, ohne dass die jeweiligen Wassergesetze bzw. das WHG verändert werden müssten. Wird ein Gewässer von Amphibien zu Reproduktionszwecken genutzt und

[166] http://ec.europa.eu/food/plant/pesticides/approval_active_substances/docs/wrkdoc10_en.pdf.

die umliegende landwirtschaftliche Nutzung wirkt sich negativ auf den Reproduktionserfolg aus, müssten aus artenschutzrechtlicher Sicht Maßnahmen ergriffen werden, um dies zu verhindern (beachte aber Ausnahmen nach § 45 Abs. 7 BNatSchG). Solche Maßnahmen stellen im einfachsten Falle Abstandsauflagen, also GR dar. Natürlich muss hierfür die notwendige Datengrundlage, v.a. bzgl. Amphibienvorkommen und langjährigen Populationsentwicklungen, geschaffen werden (siehe Kapitel 3.3.2.). Der Gesetzgeber hat der zuständigen (Wasser)Behörde mit § 38 Abs. 3 Nr. 2 WHG die Möglichkeit gegeben, die GR auszuweisen und die Breite der GR abweichend von den in § 38 Abs. 3 Satz 1 WHG genannten 5 m festzulegen. Folglich ist die zuständige Behörde in der Lage, im jeweiligen Einzelfall GR aus Amphibienschutzgründen auszuweisen, falls die Gewässer aufgrund „untergeordneter wasserwirtschaftlicher Bedeutung“ eigentlich von den Bestimmungen des WHG ausgeschlossen sind, und die Breite bestehender GR um ein Gewässer zu vergrößern, wenn nachgewiesen ist, dass der vorhandene Randstreifen die ökologische Funktionsfähigkeit des Gewässers als Lebensraum für Pflanzen und Tiere nicht gewährleistet. Informationen zu negativen Auswirkungen landwirtschaftlicher Nutzung auf lokale Amphibienpopulationen sollten von den zuständigen Naturschutzbehörden auf Grundlage regelmäßiger Monitoringergebnisse zur Populationsentwicklung und am besten Kontaminationslevel mit Pflanzenschutz- und Düngemitteln (was bisher für kleine Gewässer nicht durchgeführt wird[167]) gesammelt und an die zuständige (Wasser)Behörde weitergeleitet werden, welche dann aktiv werden sollte, um ggf. die Breite der benötigten GR anzupassen (oder überhaupt GR vorzuschreiben). Solche Grundlagendaten sind darüberhinaus streng genommen auch notwendig, um Ausnahmen nach § 45 Abs. 7 BNatSchG zu erteilen.

3.2.2. Amphibienmonitoring

Wie bereits mehrfach erwähnt ist ein Monitoring von Amphibienvorkommen in der Kulturlandschaft zwingend erforderlich, um eine Datengrundlage zu schaffen, auf der Schutzmaßnahmen erfolgen können. Eine standardisierte Methodik für ein solches Amphibienmonitoring in der Agrarlandschaft findet sich etwa in BÖLL *et al.* (2013, S. 39 ff.). Ein solches Monitoring sollte zeitnah zumindest in den Natura 2000-Gebieten beginnen, welche für Amphibienarten des Anhangs II der FFH-Richtlinie ausgewiesen wurden und

[167] NAP, S. 13.

aufgrund der Landnutzung in den Schutzgebieten sowie ihrer Biologie und Ökologie ein erhöhtes Expositionsrisiko gegenüber Agrochemikalien besitzen (WAGNER *et al.* 2014, S. 69 ff.). Diese Tatsachen sprechen u.a. für die Einrichtung einer bundesweiten Koordinationsstelle für den Amphibienschutz, wie es sie etwa in der Schweiz bereits gibt (www.karch.ch).

3.2.3. Gewässerrandstreifen und Mindererträgsareale für den Amphibienschutz

Die GR sollten über eine standortspezifische Bepflanzung verfügen, um effektiv vor schädlichen Stoffeinträgen in die Gewässer zu schützen (vgl. Kapitel 1.1.) und terrestrischen Lebensraum zu bieten (zudem sind auch etwa einfache Totholzhaufen sehr wichtig für Erd- und Wechselkröten: INDERMAUR & SCHMIDT 2011, S. 2548 ff.). Für manche Agrochemikalien scheinen 5-10 m Abstand zu genügen, um schädliche Pflanzenschutz- und Düngemitteleinträge zu verhindern (vgl. auch Tabelle 1), für andere (besonders manche Insektizide) sollten notwendige Abstandsauflagen „amphibienspezifisch“ überprüft und evtl. angepasst werden (WAGNER & HENDLER, eingereicht). Würde aber der Anspruch erhoben werden, die gesamten terrestrischen Lebensräume um die Gewässer mitzuschützen, können effektive GR bis zu 300 m Abstand für manche Amphibienarten betragen (siehe SEMLITSCH & BODIE 2002, S. 1219 ff.). Ob solche Werte in der Praxis umsetzbar sind, erscheint mehr als fraglich, v.a. wenn es sich um Kleinstgewässer in der Agrarlandschaft handelt (mit großer Wahrscheinlichkeit würde dies zu einem erheblichen wirtschaftlichen Schaden eines Betriebes respektive Landwirtes führen, vgl. §45 Abs. 7 BNatSchG). Prinzipiell sollte aus naturschutzfachlicher Sicht darauf geachtet werden, dass eine Population einer streng geschützten Amphibienart, welche in der Agrarlandschaft persistiert, keinen rückläufigen Bestand aufweist und schließlich ausstirbt, auch wenn Einzeltiere durch landwirtschaftliche Nutzung getötet werden. Hierzu bemerken etwa BERGER *et al.* (2011, S. 1 ff.), dass sich intensive und ertragreiche Produktion und erhöhte Biodiversität auf derselben Fläche prinzipiell ausschließen, schlagen jedoch vor, <u>Mindererträgsareale für den Naturschutz zu reservieren</u>. So könnten die Bestände gestärkt werden, um den Verlust von Einzeltieren durch die Bewirtschaftung zu kompensieren. Auf diesen Flächen könnten auch optimal auf die vorkommenden Arten abgestimmte Laichgewässer und Landlebensräume (welche aufgrund der oft hohen Raumnutzung der Arten jedoch wohl nur Teilhabitate darstellen können) angelegt werden (evtl. im Rahmen des Vertragsnaturschutzes durchführbar), welche den Reproduktionserfolg der Population erhöhen und eventuell Tiere davon abhalten, etwa überflutete Flächen auf der intensiv genutzten Landwirtschaftsfläche zu nutzen. Im Falle der Beeinträchtigung terrestrischer Lebensstadien auf der Nutzfläche sollten Naturschutzbehörden jährlich und kleinräumig für landwirtschaftlich genutzte Gebiete mit Amphibienvorkommen einen Zeitraum festlegen, in denen es zu Wanderbewegungen der dortigen Arten kommt, und diesen den ansässigen Landwirten mitteilen. Der Einsatz von Agrochemikalien (aber auch mechanische Bodenbearbeitung) sollte in dieser Zeit auf das Minimum reduziert werden, welches nicht betriebsschädigend wirkt. Dies muss jedoch im Einzelfall entschieden werden[168].

4. Zusammenfassung des rechtswissenschaftlichen Teils

Die einheimischen Amphibien stellen eine Artengruppe mit besonders hohem natur- und artenschutzfachlichen Wert dar. Landwirtschaftliche Nutzung und im Speziellen die Ausbringung von Pflanzenschutz- und Düngemitteln kann sich negativ auf Amphibienvorkommen auswirken, mit Sicherheit auf Individuen. Amphibien kommen oftmals nicht wegen landwirtschaftlicher Nutzung in der Kulturlandschaft vor, wie viele andere Offenlandarten, sondern sie persistieren in ihr, da große Teile der Primärlebensräume (besonders Auen) vieler Arten für die landwirtschaftliche Nutzung zerstört wurden. Während die terrestrischen Lebensstadien (Juvenile, Adulti) v.a. durch direkten Kontakt mit Agrochemikalien gefährdet sind oder ihre Nahrungsgrundlage durch bestimmte Pestizideinsätze verringert wird, stellen schädliche Stoffeinträge in Laichgewässer die größte Gefahr für die aquatischen Lebensstadien (Embryonen, Larven) dar. Grundsätzlich sollen Gewässerrandstreifen u.a. adverse Effekte auf aquatische Organismen verhindern. Jedoch werden viele Amphibienlaichgewässer auf rechtlicher Grundlage von Schutzbestimmungen ausgeschlossen und es bestehen hier keinerlei Abstandsauflagen. Dies kann bei der Applikation bestimmter Agrochemikalien zu Konzentrationen in den Gewässern führen, die sich stark negativ auf den Reproduktionserfolg und damit eventuell die Populationsentwicklung auswirken. Es ist zudem zu befürchten, dass für manche Amphibienarten bereits als unbedenklich geltende Konzentrationen (auf Grundlage der Ergebnisse mit Standardtestorganismen), welche trotz bestehender GR in die Gewässer

[168] Siehe Fn. 163.

gelangen, Schadpotenzial besitzen. Besonders auf Grundlage artenschutzrechtlicher Vorgaben können aber GR um Amphibiengewässer ausgewiesen und deren Breite standortspezifisch festgelegt werden. Dafür ist jedoch eine gute Datengrundlage zu Amphibienvorkommen, deren Bestandsentwicklungen sowie Habitatkontamination mit Agrochemikalien notwendig, welche derzeit aber noch nicht vorhanden ist. Zudem sollten die Effekte mancher Pestizide durch spezielle amphibientoxikologische Studien überprüft und auf Grundlage dieser Ergebnisse Mindestabstände festgelegt werden. Da es in der Praxis jedoch kaum möglich ist, genügend breite GR als Schutzmaßnahme um jedes von Amphibien genutzte Kleinstgewässer wie etwa Entwässerungsgräben anzulegen und jegliche Pestizideinsätze streng zu regulieren – vor allen Dingen, weil Ausnahmen von den Verbotstatbeständen des § 44 BNatSchG seit der Kleinen Novelle 2007 bereits möglich sind, sobald ein Einzelbetrieb erheblichen wirtschadlichen Schaden erfahren würde – sollten lokale Vorkommen durch gezielte Artenschutzmaßnahmen (Naturschutzbrachen in Minderertragsarealen, Anlage und Pflege artspezifischer Land- und Wasserlebensräume) gestärkt werden, um zumindest negativen Effekten auf der Populationsebene entgegenzuwirken.

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Anhänge

Appendix A: Overview on studies concerning impacts of GLY and GBH on different amphibian species [sorted alphabetically by species]

Species	Family	Order	Region	Developmental Gosner stages at the beginning of the test	Study type	Tested GBH, GLY or surfactant	Endpoints	Maximum tested concentration (when named)	LC50, LD50 or EC50 (when available)	Renewal each	Duration	Important co-factors	Main conclusion	Reference
Ambystoma gracile	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	2.8 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Ambystoma gracile	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	1.4 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; salamanders more resistant than anurans	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.

Ambystoma laterale	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	3.2 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Ambystoma macrodactylum	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	1.2 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; salamanders more resistant than anurans	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.
Ambystoma maculatum	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	2.8 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Ambystoma maculatum	Ambysto-matidae	Uro-dela	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	No effects; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.

Ambystoma tigrinum	Ambysto-matidae	Uro-dela	Nearctic	Larvae	Field	Accord (NPE surfactant)	Survival, development, and behavior	2.0 mg a.e./L		No renewal	1,176 h		Significant effects, but less risk than other GBH	Brodman R, Newman WD, Laurie K, Osterfeld S, Lenzo N. 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. J Herpetol 44: 69-82.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup Weed & Grasskiller	Survival	2.85 mg a.e./L		No renewal	480 h	No soil, sand or loam soil; always rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	100% mortality; no higher survival due to soil presence	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.6 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.

Anaxyrus americanus	Bufonidae	Anura	Nearctic	Embryos	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	4.8 mg a.e./L (pH 6.0); 6.4 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Embryos more resistant; higher toxicity with higher pH level	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	2.9 mg a.e./L (pH 6.0); 1.7 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Higher toxicity with higher pH level, if pH ≥ 7.5, LC50 at or below EEC (1.44 mg a.e./L)	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.

Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup WeatherMAX	Survival, body mass and time to metamorphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished metamorphosis		No effects on survival and mass, but significant effects on time to metamorphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	Survival, body mass and time to metamorphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished metamorphosis		No effects on survival and mass, but significant effects on time to metamorphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup (POEA surfactant)	Survival and biomass	0.98 mg a.e./L		No renewal	552 h	3 predator treatments (no, newts, larval beetles) crossed with 3 pesticide treatments (no, malathion, Roundup)	No effect of Roundup; high mortality with predators	Relyea RA, Schoeppner NM, Hoverman JT. 2005. Pesticides and amphibians: the importance of community context. Ecol Appl 15: 1125-1134.

Anaxyrus americanus	Bufonidae	Anura	Nearctic	Larvae	Field	Accord (NPE surfactant)	Survival, development, and behavior	2.0 mg a.e./L		No renewal	1,176 h	Predators (*Ambystoma tigrinum*)	Significant effects, but less risk than other GBH	Brodman R, Newman WD, Laurie K, Osterfeld S, Lenzo N. 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. J Herpetol 44: 69-82.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	No effects; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.
Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage26)	Meso-cosm	Roundup OriginalMAX	Survival and development	3 mg a.e./L		Applications at different days (singely and cumulatively)	432 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Earlier application caused higher mortality; single large applications had larger effects than multiple small ones of the same amount	Jones DK, Hammond JI, Relyea RA. 2010. Roundup® and amphibians: the importance of concentration, application time, and stratification. Environ Toxicol Chem 29: 2016-2025.

Anaxyrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	Survival and growth	2 mg a.e/L		96 h	384 h	Roundup, carbaryl (Sevin), diazinon and malathion were tested alone and in pairwise combination	Significant mortality and reduced growth due to Roundup; the effects of combined pesticides were never larger than the more deadly of the two pesticides alone at 2 mg/L.	Relyea RA. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environ Toxicol Chem 23: 1737-1742.
Anaxyrus boreas	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	2.0 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Anaxyrus boreas	Bufonidae	Anura	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	1.5 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; total mortality in the highest treatment after 24 h	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.

Anaxyrus cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Super Concentrate	Survival	1.3 mg a.e./m²		No renewal	48 h	Moist paper towel	Significant effect	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Anaxyrus cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Super Concentrate	Survival	1.3 mg a.e./m²		No renewal	48 h	Soil	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Anaxyrus cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Ready-To-Use Plus	Survival	1.3 mg a.e./m²		No renewal	48 h	Moist paper towel	Significant effect	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Anaxyrus cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Ready-To-Use Plus	Survival	1.3 mg a.e./m²		No renewal	48 h	Soil	Significant effect	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Anaxyrus fowleri	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	4.2 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Anaxyrus fowleri	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup WeatherMA X	LC50	7 mg a.e./L	2.0 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Anaxyrus woodhousii	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed & Grasskiller	Survival	1.2 mg a.e./m^2		No renewal	24 h		Nearly 80% mortality	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.

Anaxyrus woodhousii	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		1.89 mg a.e./L	96 h	384 h	With and without predatory cues	Roundup and time with significant effects on survival; no additional effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.
Anayxrus americanus	Bufonidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	< 4 mg a.e./L	No renewal	96 h		Significant acute effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Anayxrus americanus	Bufonidae	Anura	Nearctic	Embryos (Gosner stage 20)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	8 mg a.e./L	No renewal	96 h		Significant acute effect; embryos more resistant	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.

Ascaphus truei	Leiopel-matidae	Anura	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	> 2000 mg a.e./L (intraperitoneal)	No renewal	96 h		Low intraperitoneal toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.
Bufo cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup WeatherMAX	Survival	0.2 mg a.e./m²		No renewal	48 h	Moist paper towel	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Bufo cognatus	Bufonidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup WeatherMAX	Survival	0.2 mg a.e./m²		No renewal	48 h	Soil	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Centrolene prosoblepon	Centrolenidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		4.5 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.

Centrolene prosoblepon	Centrolenidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		2.4 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Chioglossa lusitanica	Salaman-dridae	Uro-dela	Palae-arctic	Embryos	Labor-atory	Roundup Plus (POEA surfactant)	Survival, development, and size at hatching	2 mg a.e/L		168 h	2520 h	Ammonium nitrate (up to 90.3 mg/L)	No significant effect on survival and embryonic development; positive effect of Roundup Plus on size at hatching	Ortiz-Santaliestra ME, Fernandez-Beneitez MJ, Lizana M, Marco A. 2011. Influence of a combination of agricultural chemicals on embryos of the endangered Gold-striped salamander (*Chioglossa lusitanica*). Arch Environ Contam Toxicol 60: 672-680.

Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L		24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Meta-morphs	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L		24 h	48 h		An order of magnitude less sensitive than tadpoles	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Adults	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L	83.6 mg a.e./L	24 h	48 h		An order of magnitude less sensitive than tadpoles	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate isopropy-lamine salt	LC50	400 mg a.e./L	> 466 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	3.6 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Touchdown	LC50	400 mg a.e./L	9.0 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Biactive	LC50	400 mg a.e./L	> 494 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Meta-morphs	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	51.8 mg a.e./L	24 h	48 h		An order of magnitude less sensitive than tadpoles	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Adults	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	49.4 mg a.e./L	24 h	48 h		An order of magnitude less sensitive than tadpoles	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Crinia insignifera	Myobatrachidae	Anura	Australasia	Embryos	Laboratory	Teric GN8 (100% nonylphenol polyethoxylate surfactant, NPE)	Survival (LC50), malformation (EC50), teratogenicity indices (TI), and minimum concentration to inhibit growth (MCIG)	Standard method FETAX (8 mg/L NPE)	LC50 6.4, EC50 4.5, MCIG 4.0 mg/L NPE, TI 1.4	24 h	Until survivors reached G 24 (equivalent to Nieuwkoop and Faber stage 46) = 134 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Crinia insignifera	Myobatrachidae	Anura	Australasia	Early larvae (Gosner stage25)	Laboratory	Teric GN8 (100% nonylphenol polyethoxylate surfactant, NPE)	Narcosis (EC50)		2.7 (mild), 3.8 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Crinia insignifera	Myobatrachidae	Anura	Australasia	Early larvae (Gosner stage25)	Laboratory	Agral 600 (60% NPE and unspecified concentrations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		2.7 (mild), 3.5 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Crinia insignifera	Myobatra-chidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)		5.3 (mild), 6.0 (full narcosis) mg/L alcohol alkoxylate	24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Dendrobates truncatus	Dendro-batidae	Anura	Neo-tropic	Adults	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		>7.4 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Dendropsophus microcephalus	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		1.2 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.

Dicamptodon ensatus	Ambysto-matidae	Uro-dela	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	< 2000 mg a.e./L (intraperitoneal)	No renewal	96 h		Low intraperitoneal toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.
Engystomops pustulosus	Leiuperidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		19.6 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.

Engystomops pustulosus	Leiuperidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		2.8 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Ensatina eschscholtzii	Plethodon-tidae	Uro-dela	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	1070 mg a.e./L (intraperitone al)	No renewal	96 h		Low intraperitonea l toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.
Heleioporus eyrei	Lymnodyna s-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L		24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Heleioporus eyrei	Lymnodyna s-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate isopropyl-amine salt	LC50	400 mg a.e./L	> 373 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Heleioporus eyrei	Lymnodyna s-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	6.3 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Heleioporus eyrei	Lymnodyna s-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Touchdown	LC50	400 mg a.e./L	16.1 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Heleioporus eyrei	Lymnodynas-tidae	Anura	Australasia	Early larvae (Gosner stage25)	Labor-atory	Roundup Biactive	LC50	400 mg a.e./L	> 427 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Heleioporus eyrei	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)			24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Heleioporus eyrei	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Agral 600 (60% NPE and unspecified concen-trations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		> 10.6 (mild), 12.1 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Heleioporus eyrei	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)		< 10.0 (mild), 25.4 (full narcosis) mg/L alcohol alkoxylate	24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Hyla chrysocelis	Hylidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	2.5 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Hyla chrysocelis	Hylidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Weather-MAX	LC50	7 mg a.e./L	3.3 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.

Hyla chrysocelis	Hylidae	Anura	Nearctic	Adults	Field	Roundup Weed & Grasskiller	Oviposition site selection	2.4 mg a.e./L		No renewal	One night (about 12 h)	With and without predators	Complete avoidance as breeding pond	Takahashi M. 2007. Oviposition site selection: pesticide avoidance by Gray treefrogs. Environ Toxicol Chem 26: 1476-1480.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup Weed & Grasskiller	Survival	2.85 mg a.e./L		No renewal	480 h	No soil, sand or loam soil; always rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Nearly 100% mortality; no higher survival due to soil presence	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.
Hyla versicolor	Hylidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed & Grasskiller	Survival	1.2 mg a.e./m²		No renewal	24 h		Nearly 80% mortality	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		1.01 mg a.e./L	96 h	384 h	With and without predatory cues	Roundup and time with significant effects on survival; no additional effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.

Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labo-ratory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.7 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup WeatherMAX	Survival, body mass and time to meta-morphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished metamorphosis		Significant effect on survival (about 80% mortality); no effects on mass and time to metamorphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	Survival, body mass and time to meta-morphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished metamorphosis		No effects on survival, mass, and time to metamorphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.

Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup (POEA surfactant)	Survival and biomass	0.98 mg a.e./L		No renewal	552 h	3 predator treatments (no, newts, larval beetles) crossed with 3 pesticide treatments (no, malathion, Roundup)	Without predators nearly 70% mortality; high mortality with predators	Relyea RA, Schoeppner NM, Hoverman JT. 2005. Pesticides and amphibians: the importance of community context. Ecol Appl 15: 1125-1134.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	100% mortality; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup OriginalMAX	Survival (LC50) and development	3 mg a.e./L	2.0 (low), 2.3 (medium), 1.7 (high competition) mg a.e./L	No renewal	528 h	Low/medium/high tadpole density; rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Similar mortality across densities	Jones DK, Hammond JI, Relyea RA. 2011. Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. Environ Toxicol Chem 30: 446-454.

Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae	Meso-cosm	Glyphosate (if salt or acid not named)	Survival, size and time to meta-morphosis	6.9 p.p.b.		No renewal	1368 h	Different herbicides and insecticides tested singly and in combinations; rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	No single effects of glyphosate and no mortality due to any treatment; growth nearly doubled when all pesticides and all insecticides were combined due to reduced competition	Relyea RA. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159: 363-376.
Hyla versicolor	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	Survival and growth	2 mg a.e/L		96 h	384 h	Roundup, carbaryl (Sevin), diazinon and malathion were tested alone and in pairwise combination	No significant mortality or reduced growth due to Roundup; the effects of combined pesticides were never larger than the more deadly of the two pesticides alone at 2 mg/L.	Relyea RA. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environ Toxicol Chem 23: 1737-1742.
Hyla versicolor	Hylidae	Anura	Nearctic	Adults	Field	Roundup Weed & Grasskiller	Oviposition site selection	2.4 mg a.e./L		No renewal	One night (about 12 h)	With and without predators	Complete avoidance as breeding pond	Takahashi M. 2007. Oviposition site selection: pesticide avoidance by Gray treefrogs. Environ Toxicol Chem 26: 1476-1480.

Hypsiboas crepitans	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		7.3 mg a.e./L	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Hypsiboas crepitans	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		2.1 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Limnodynastes dorsalis	Lymnodyna-s-tidae	Anura	Austra-lasia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L		24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylamine salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate isopropyl-amine salt	LC50	400 mg a.e./L	> 400 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	3.0 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylamine salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Touchdown	LC50	400 mg a.e./L	12.0 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylamine salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Biactive	LC50	400 mg a.e./L	> 400 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyeth-oxylate surfactant, NPE)	Narcosis (EC50)			24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Agral 600 (60% NPE and unspecified concen-trations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		4.1 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Limnodynastes dorsalis	Lymnodynas-tidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)		< 6.0 (mild), 14.3 (full narcosis) mg/L alcohol alkoxylate	24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Lithobates blairi	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labo-ratory	Kleeraway Grass & weed Killer RTU (ethoxylated tallowamine surfactant)	Survival	0.1 concentration (1 part herbicide/9 parts deionized water)		No renewal	24 h		Significant mortality, total mortality at 0.1-0.001 concentrations	Smith GR. 2001. Effects of acute exposure to a commercial formulation of glyphosate on the tadpoles of two species of anurans. B Environ Contam Tox 67: 483-488.
Lithobates blairi	Ranidae	Anura	Nearctic	Larvae (Gosner stage26-30)	Labor-atory	Kleeraway Grass & weed Killer RTU (ethoxylated tallowamine surfactant)	Survival	0.1 concentration (1 part herbicide/9 parts deionized water)		No renewal	24 h		Significant mortality with total mortality at all concentrations	Smith GR. 2001. Effects of acute exposure to a commercial formulation of glyphosate on the tadpoles of two species of anurans. B Environ Contam Tox 67: 483-488.
Lithobates blairi	Ranidae	Anura	Nearctic	Survivors from the first experiment at 0.0001 concen-trations	Labor-atory	Kleeraway Grass & weed Killer RTU (ethoxylated tallowamine surfactant)	Development	0.0001 concentration		No renewal	336 h		No effects on development (final mass and Gosner-stage)	Smith GR. 2001. Effects of acute exposure to a commercial formulation of glyphosate on the tadpoles of two species of anurans. B Environ Contam Tox 67: 483-488.

Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		1.55 mg a.e./L	96 h	384 h	With and without predatory cues	Roundup and time with significant effects on survival; no additional effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.
Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	0.8 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory		Oxidative stress, clinical signs, and cardiac function	0.27 mg a.e./L		No renewal	48 h		Significant increase of oxidative stress in liver and muscle tissue; increased acivitiy and heart rate	Costa M, Monteiro D, Oliveira-Neto A, Rantin F, Kalinin A. 2008. Oxidative stress biomarkers and heart function in Bullfrog tadpoles exposed to Roundup Original®. Ecotoxicology 17: 153-163.

Lithobates catesbeianus	Ranidae	Anura	Nearctic	Larvae	Labor-atory	Roundup Original (POEA surfactant)	DNA damage	1.7 mg a.e./L		No renewal	24 h		Significant DNA damage	Clements C, Ralph S, Petras M. 1997. Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Environ Mol Mutagen 29: 277-288.
Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	2.8 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.

Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup WeatherMAX	LC50	7 mg a.e./L	2.0 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup OriginalMAX	Survival (LC50) and development	3 mg a.e./L	2.2 (low), 2.1 (medium), 1.6 (high competition) mg a.e./L	No renewal	528 h	Low/medium/high tadpole density; rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Increased mortality with increased density	Jones DK, Hammond JI, Relyea RA. 2011. Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. Environ Toxicol Chem 30: 446-454.
Lithobates catesbeianus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	Survival and growth	2 mg a.e/L		96 h	384 h	Roundup, carbaryl (Sevin), diazinon and malathion were tested alone and in pairwise combination	Significant mortality and reduced growth due to Roundup; the effects of combined pesticides were never larger than the more deadly of the two pesticides alone at 2 mg/L.	Relyea RA. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environ Toxicol Chem 23: 1737-1742.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Field	Vision (POEA surfactant)		Aerial application (direct, adjacent, and vegetation bufferd wetlands); 1.95 mg a.e./L (directly over-sprayed wetland)		No renewal	96 h	Larvae in enclusores in natural wetlands; buffered wetlands (max. 0.31 mg a.e./L, mean 0.03 mg a.e./L) served as control	No significant differences in mortality between directly oversprayed, adjacent, and buffered wetlands	Thompson DG, Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian larvae in forest wetlands. Environ Toxicol Chem 23: 843-849.
Lithobates clamitans	Ranidae	Anura	Nearctic	Juveniles	Field	VisionMAX	Survival, body condition, liver somatic index, *Bd* infection	4.3 kg a.e./ha		No renewal	336 h	Test in a natural wetland	No significant effects	Edge CB, Gahl MK, Pauli BD, Thompson DG, Houlahan JE. 2011. Exposure of juvenile Green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide. Ecotox Environ Safe 74: 1363-1369.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		1.63 mg a.e./L	96 h	384 h	With and without predatory cues	Roundup and time with significant effects on survival; no additional effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.4 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Field	Vision (POEA surfactant)	Survival, growth, and avoidance response	14.3 mg a.e./L	2.70-4.34 mg a.e./L	No renewal	96 h	Larvae in enclusores in natural wetlands	At EEC (1.4 mg a.e./L) no effects on avoidance response and mortality, growth either equivalent or higher than controls; above EEC significant effects	Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR, Thompson DG. 2004. Effects of Vision® herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. Environ Toxicol Chem 23: 832-842.

Lithobates clamitans	Ranidae	Anura	Nearctic	Embryos	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	5.3 mg a.e./L (pH 6.0); 4.1 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Embryos more resistant; higher toxicity with higher pH level	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	3.5 mg a.e./L (pH 6.0); 1.4 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Higher toxicity with higher pH level, if pH ≥ 7.5, LC50 at or below EEC (1.44 mg a.e./L)	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	2.0 mg a.e./L	No renewal	96 h		Significant acute effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Embryos (Gosner stage 20)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	7.1 mg a.e./L	No renewal	96 h		Significant acute effect; embryos more resistant	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Glyphosate isopropyl-amine salt	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	> 17.9 mg a.e./L	96 h	1008 h		No significant acute nor chronic effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Laboratory	POEA	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg/L	2.2 mg a.e./L	96 h	1008 h		Significant acute and chronic effect (smaller size at and increased time to metamorphosis, tail damage, gonadal abnormalities, elevated thyroid hormone receptor b mRNA transcript levels	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Biactive	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	> 17.9 mg a.e./L	96 h	1008 h		No significant acute nor chronic effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Touchdown	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	> 17.9 mg a.e./L	96 h	1008 h		No significant acute nor chronic effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Glyfos BIO	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	> 17.9 mg a.e./L	96 h	1008 h		No significant acute nor chronic effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Glyfos AU	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	8.9 mg a.e./L	96 h	1008 h		Significant acute effect, but no significant chronic effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Transorb (POEA surfactant)	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	2.2 mg a.e./L	96 h	1008 h		Significant acute and chronic effect (smaller size at and increased time to metamorphosis, tail damage, gonadal abnormalities, elevated thyroid hormone receptor b mRNA transcript levels	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	Survival (LC50), develop-ment, gonads, sex, mRNA expression	1.8 mg a.e./L	2.0 mg a.e./L	96 h	1008 h		Significant acute and chronic effect (smaller size at and increased time to metamorphosis, tail damage, gonadal abnormalities, elevated thyroid hormone receptor b mRNA transcript levels	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	4.2 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Weather-MAX	LC50	7 mg a.e./L	2.8 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Lithobates clamitans	Ranidae	Anura	Nearctic	Larvae	Field	Accord (NPE surfactant)	Survival, develop-ment, and behavior	2.0 mg a.e./L		No renewal	1,176 h	Predators (*Ambystoma tigrinum*)	Significant effects, but less risk than other GBH	Brodman R, Newman WD, Laurie K, Osterfeld S, Lenzo N. 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. J Herpetol 44: 69-82.

Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup OriginalMAX	Survival (LC50) and development	3 mg a.e./L	2.6 (low), 2.4 (medium), 2.2 (high competition) mg a.e./L	No renewal	528 h	Low/medium/high tadpole density; rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Similar mortality across densities	Jones DK, Hammond JI, Relyea RA. 2011. Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. Environ Toxicol Chem 30: 446-454.
Lithobates clamitans	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	Survival and growth	2 mg a.e/L		96 h	384 h	Roundup, carbaryl (Sevin), diazinon and malathion were tested alone and in pairwise combination	Significant mortality and reduced growth due to Roundup; the effects of combined pesticides were never larger than the more deadly of the two pesticides alone at 2 mg/L.	Relyea RA. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environ Toxicol Chem 23: 1737-1742.
Lithobates clamitans	Ranidae	Anura	Nearctic	Larvae (Gosner stage24-26)	Labor-atory	Glyphosate (if salt or acid not named)	Cercarial infectivity and survival	3.7 mg a.e./L		One renewal after 24 h	336 h	*Echinostoma trivolvis* cercariae	Higher infection rate, especially at younger larvae; no synergistic effect of pesticide and cercariae on survival (in a second experiment, exposure of the cercariae alone did not alter their virulence)	Rohr JR, Raffel TR, Sessions SK, Hudson PJ. 2008. Understanding the net effects of pesticides on amphibian trematode infections. Ecol Appl 18: 1743-1753.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Field	Vision (POEA surfactant)	Survival	Aerial application (direct, adjacent, and vegetation bufferd wetlands); 1.95 mg a.e./L (directly over-sprayed wetland)		No renewal	96 h	Larvae in enclusores in natural wetlands; buffered wetlands (max. 0.31 mg a.e./L, mean 0.03 mg a.e./L) served as control	No significant differences in mortality between directly oversprayed, adjacent, and buffered wetlands	Thompson DG, Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian larvae in forest wetlands. Environ Toxicol Chem 23: 843-849.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Meso-cosm	Roundup Weed & Grasskiller	Survival	2.85 mg a.e./L		No renewal	480 h	No soil, sand or loam soil; always rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Nearly 100% mortality; no higher survival due to soil presence	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		1.85 mg a.e./L	96 h	384 h	With and without predatory cues	Roundup and time with significant effects on survival; no additional effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.5 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Field	Vision (POEA surfactant)	Survival, growth, and avoidance response	14.3 mg a.e./L	4.25-11.47 mg a.e./L	No renewal	96 h	Larvae in enclusores in natural wetlands	At EEC (1.4 mg a.e./L) no effects on avoidance response and mortality, growth either equivalent or higher than controls; above EEC significant effects	Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR, Thompson DG. 2004. Effects of Vision® herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. Environ Toxicol Chem 23: 832-842.

Lithobates pipiens	Ranidae	Anura	Nearctic	Embryos	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	15.1 mg a.e./L (pH 6.0); 7.5 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Embryos more resistant; higher toxicity with higher pH level	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	1.8 mg a.e./L (pH 6.0); 1.1 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Higher toxicity with higher pH level, if pH ≥ 7.5, LC50 at or below EEC (1.44 mg a.e./L)	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	2.9 mg a.e./L	No renewal	96 h		Significant acute effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates pipiens	Ranidae	Anura	Nearctic	Embryos (Gosner stage 20)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	6.5 mg a.e./L	No renewal	96 h		Significant acute effect; embryos more resistant	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae	Labor-atory	Rodeo (without surfactant)	LC50		6.5 mg a.e./L	48 h	96 h	LC50 values calculated from one experiment using the Rodeo/surfacta nt mixture	Toxicity is largely determined by the concentration of R-11	Trumbo J. 2005. An assessment of the hazard of a mixture of the herbicide Rodeo® and the non-ionic surfactant R-11® to aquatic invertebrates and larval amphibians. Calif Fish Game 91: 38-46.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae	Labor-atory	R-11 (nonyl-phenol polyethoxy-late surfactant, NPE)	LC50		1.7 mg a.e./L	48 h	96 h	LC50 values calculated from one experiment using the Rodeo/surfacta nt mixture	Toxicity is largely determined by the concentration of R-11	Trumbo J. 2005. An assessment of the hazard of a mixture of the herbicide Rodeo® and the non-ionic surfactant R-11® to aquatic invertebrates and larval amphibians. Calif Fish Game 91: 38-46.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	1.8 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Weather-MAX	LC50	7 mg a.e./L	2.3 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Lithobates pipiens	Ranidae	Anura	Nearctic	Late larvae	Meso-cosm	Roundup (POEA surfactant)	Survival and biomass	0.98 mg a.e./L		No renewal	552 h	3 predator treatments (no, newts, larval beetles) crossed with 3 pesticide treatments (no, malathion, Roundup)	Without predators nearly 30% mortality; high mortality with predators and additional 20% reduction with Roundup	Relyea RA, Schoeppner NM, Hoverman JT. 2005. Pesticides and amphibians: the importance of community context. Ecol Appl 15: 1125-1134.
Lithobates pipiens	Ranidae	Anura	Nearctic	Larvae	Field	Accord (NPE surfactant)	Survival, develop-ment, and behavior	2.0 mg a.e./L		No renewal	1,176 h	Predators (*Ambystoma tigrinum*)	Significant effects, but less risk than other GBH	Brodman R, Newman WD, Laurie K, Osterfeld S, Lenzo N. 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. J Herpetol 44: 69-82.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	100% mortality; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.
Lithobates pipiens	Ranidae	Anura	Nearctic	Late larvae	Meso-cosm	Glyphosate (if salt or acid not named)	Survival, size and time to meta-morphosis	6.9 p.p.b.		No renewal	1368	Different herbicides and insecticides tested singely and in combinations; rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	No single effects of glyphosate; nearly 100% mortality when all pesticides and all insecticides were combined	Relyea RA. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159: 363-376.
Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Vision (POEA surfactant)	Survival	1.5 mg a.e./L		24 h	240 h	pH 5.5/pH 7.5, low/high food level	Significant effects, mainly with high pH	Chen CY, Hathaway KM, Folt CL. 2004. Multiple stress effects of Vision® herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. Environ Toxicol Chem 23: 823-831.

Lithobates pipiens	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	Survival and growth	2 mg a.e/L		96 h	384 h	Roundup, carbaryl (Sevin), diazinon and malathion were tested alone and in pairwise combination	No significant mortality or reduced growth due to Roundup; the effects of combined pesticides were never larger than the more deadly of the two pesticides alone at 2 mg/L.	Relyea RA. 2004. Growth and survival of five amphibian species exposed to combinations of pesticides. Environ Toxicol Chem 23: 1737-1742.
Lithobates sphenocephalus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	7 mg a.e./L	2.1 mg a.e./L	No renewal	96 h		Differences between species; little risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.

Lithobates sphenocephalus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Weather-MAX	LC50	7 mg a.e./L	1.3 mg a.e./L	No renewal	96 h		Differences between species; risk concerning EEC (1.1 mg a.e./L)	Fuentes L, Moore LJ, Rodgers JH, Jr., Bowerman WW, Yarrow GK, Chao WY. 2011. Comparative toxicity of two glyphosate formulations to six North American larval anurans. Environ Toxicol Chem 30: 2756-2761.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 26)	Labor-atory	Roundup Weed and Grass Killer Concentrate Plus	Survival	2% glyphosate		No renewal	96 h		All tadpoles exposed to ≥ 0.00098% glyphosate died within 24 h. Tadpoles exposed to ≤ 0.00049% showed high survivorship	Comstock BA, Sprinkle SL, Smith GR. 2007. Actue toxic effects of Round-Up herbicide on Wood frog tadpoles (*Rana sylvatica*). J Freshwat Ecol 22: 705-708.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed & Grasskiller	Survival	1.2 mg a.e./m²		No renewal	24 h		Nearly 65% mortality	Relyea RA. 2005. The lethal impact of Roundup® on aquatic and terrestrial amphibians. Ecol Appl 15: 1118-1124.

Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		0.99 mg a.e./L	96 h	384 h	Without predatory cues	Roundup and time with significant effects on survival	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup (POEA surfactant)	LC50		0.41 mg a.e./L	96 h	384 h	With predatory cues	Additional significant effect of predatory cues	Relyea RA. 2005. The lethal impacts of Roundup® and predatory stress on six species of North American tadpoles. Arch Environ Contam Toxicol 48: 351-357.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.9 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.

Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage 25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	5.1 mg a.e./L	No renewal	96 h		Significant acute effect	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Embryos (Gosner stage 20)	Labor-atory	Roundup Original (POEA surfactant)	LC50	8 mg a.e./L	> 8 mg a.e./L	No renewal	96 h		Significant acute effect; embryos more resistant	Howe CM, Berrill M, Pauli BD, Helbing CC, Werry K, Veldhoen N. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environ Toxicol Chem 23: 1928-1938.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	Nearly 100% mortality; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.

Lithobates sylvaticus	Ranidae	Anura	Nearctic	Early larvae (Gosner stage26)	Meso-cosm	Roundup OriginalMAX	Survival and development	3 mg a.e./L		Applications at different days (singely and cumulatively)	432 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton	Earlier application caused higher mortality; single large applications had larger effects than multiple small ones of the same amount	Jones DK, Hammond JI, Relyea RA. 2010. Roundup® and amphibians: The importance of concentration, application time, and stratification. Environ Toxicol Chem 29: 2016-2025.
Lithobates sylvaticus	Ranidae	Anura	Nearctic	Late larvae (Gosner stage 32)	Labor-atory	Roundup Weather-MAX	Survival, development, infection rate with *Bd*	2.9 mg a.e./L		No renewal	960 h	Two strains of *Bd*	No effect on survival and development; less GBH exposed animals with *Bd* infection	Gahl MK, Pauli BD, Houlahan JE. 2011. Effects of chytrid fungus and a glyphosate-based herbicide on survival and growth of Wood frogs (*Lithobates sylvaticus*). Ecol Appl 21: 2521-2529.
Litoria adelaidensis	Hylidae	Anura	Austral-asia	Embryos	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Survival (LC50), malform-ation (EC50), terato-genicity indices (TI), and minimum concen-tration to inhibit growth (MCIG)	Standard method FETAX (8.2 mg/L NPE)	MCIG 5.1 mg/L NPE	24 h	Until survivors reached G 24 (equivalent to Nieuwkoop and Faber stage 46) = 140 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.

Litoria adelaidensis	Hylidae	Anura	Australasia	Embryos	Laboratory	Teric GN8 (100% nonylphenol polyethoxylate surfactant, NPE)	Survival (LC50), malformation (EC50), teratogenicity indices (TI), and minimum concentration to inhibit growth (MCIG)	Standard method FETAX (8.2 mg/L NPE)	MCIG 2.0 mg/L NPE	24 h	Until survivors reached G 24 (equivalent to Nieuwkoop and Faber stage 46) = 140 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Litoria adelaidensis	Hylidae	Anura	Australasia	Embryos	Laboratory	Teric GN8 (100% nonylphenol polyethoxylate surfactant, NPE)	Survival (LC50), malformation (EC50), teratogenicity indices (TI), and minimum concentration to inhibit growth (MCIG)	Standard method FETAX (9.6 mg/L NPE)	LC50 9.2, EC50 8.8 mg/L NPE, TI 1.0	24 h	Until survivors reached G 24 (equivalent to Nieuwkoop and Faber stage 46) = 140 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Litoria adelaidensis	Hylidae	Anura	Australasia	Embryos	Laboratory	Teric GN8 (100% nonylphenol polyethoxylate surfactant, NPE)	Survival (LC50), malformation (EC50), teratogenicity indices (TI), and minimum concentration to inhibit growth (MCIG)	Standard method FETAX (10 mg/L NPE)	LC50 9.2, EC50 8.8, MCIG 6.0 mg/L NPE, TI 1.0	24 h	Until survivors reached G 24 (equivalent to Nieuwkoop and Faber stage 46) = 140 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.

Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate acid	LC50	400 mg a.e./L	81.2 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Glyphosate isopropyl-amine salt	LC50	400 mg a.e./L	> 343 mg a.e./L	24 h	48 h		No mortality	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Original (POEA surfactant)	LC50	400 mg a.e./L	2.9 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.

Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Touchdown	LC50	400 mg a.e./L		24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Roundup Biactive	LC50	400 mg a.e./L	328 mg a.e./L	24 h	48 h		General acute toxicity Roundup > Touchdown > glyphosate acid > Roundup Biactive > glyphosate isopropylami ne salt	Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of Southwestern Australian frogs. Arch Environ Contam Toxicol 36: 193-199.
Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)			24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	Agral 600 (60% NPE and unspecified concen-trations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		4.6 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Litoria moorei	Hylidae	Anura	Austral-asia	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)		< 11.0 (mild narcosis) mg/L alcohol alkoxylate	24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Notophthalmus viridescens	Salaman-dridae	Uro-dela	Nearctic	Early larvae	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	2.7 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.

Odontophrynus cordobae	Cycloram-phidae	Anura	Neo-tropic	Adults	Labor-atory	Roundup Original (POEA surfactant)	Frequency of MNE after 5 d	600 mg a.e./L		48 h	120 h		Significant DNA damage for *O. cordobae*	Bosch B, Mañas F, Gorla N, Aiassa D. 2011. Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. J Tox Env Health Sci 3: 155-163.
Pelophylax kl. *esculentus*	Ranidae	Anura	Palae-arctic	Ventral skin	Labor-atory	Glyphosate acid	Percutan-eous passage	20 ml of a special test solution with marked herbicides was used		No renewal	6 h		Flux of glyphosate was lower than of two other herbicides (atrazine and paraquat); flux was always much higher in frog than in co-tested pig ear skin	Quaranta A, Bellantuono V, Cassano G, Lippe C. 2009. Why amphibians are more sensitive than mammals to xenobiotics. PLoS ONE 4: e7699.
Pelophylax kl. *esculentus*	Ranidae	Anura	Palae-arctic	Ovarian tissue and testis	Labor-atory	Glyphosate (if salt or acid not named)	Testosterone and 17β-estradiol production	0.01 M		No renewal	6 h		No effect on gonadal steroidogenesis	Quassinti L, Maccari E, Murri O, Bramucci M. 2009. Effects of paraquat and glyphosate on steroidogenesis in gonads of the frog *Rana esculenta* in vitro. Pestic Biochem Phys 93: 91-95.

Plethodon vehiculum	Plethodon-tidae	Uro-dela	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	1170 mg a.e./L (intraperitoneal)	No renewal	96 h		Low intraperitoneal toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.
Polypedates cruciger	Rhacopho-ridae	Anura	Endemic to Sir Lanka	Early larvae (Gosner stage 25-26)	Labor-atory	Roundup Original (POEA surfactant)	LC50	25 ppm	14.99 ppm	No renewal	48 h			Jayawardena UA, Rajakaruna RS, Navaratne AN, Amerasinghe PH. 2010. Toxicity of agrochemicals to common Hourglass tree frog (*Polypedates cruciger*) in acute and chronic exposure. Int J Agr Biol 12: 641-648.

Polypedates cruciger	Rhacopho-ridae	Anura	Endemic to Sir Lanka	Early larvae (Gosner stage 25-26)	Labor-atory	Roundup Original (POEA surfactant)	Development	1 ppm		168 h	Until metamorphosis		Significant reduced survival and growth, increased time to metamorphosis; 69% malformations after 240 h	Jayawardena UA, Rajakaruna RS, Navaratne AN, Amerasinghe PH. 2010. Toxicity of agrochemicals to common Hourglass tree frog (*Polypedates cruciger*) in acute and chronic exposure. Int J Agr Biol 12: 641-648.
Pristimantis taeniatus	Straboma-tidae	Anura	Neo-tropic	Adults	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		5.6 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Pseudacris crucifer	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	0.8 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.

Pseudacris crucifer	Hylidae	Anura	Nearctic	Early larvae	Meso-cosm	Roundup Original (POEA surfactant)	Survival, biomass, species richness	3.8 mg a.e./L		No renewal	336 h	Rabbit chow, oak leaf litter, zooplankton, phytoplankton, periphyton, snails, larval damselflies, dragonflies, bettles, and hemipterans	No effects; 22% reduction of species richness; no effects on zooplankton, insect predators, or snails.	Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol Appl 15: 618-627.
Pseudacris regilla	Hylidae	Anura	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	0.2 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; total mortality in the highest treatment after 24 h; LC50 below drinking water standards	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.
Pseudacris triseriata	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Kleeraway Grass & weed Killer RTU (ethoxylated tallowamine surfactant)	Survival	0.1 concen-tration (1 part herbicide/9 parts deionized water)		No renewal	24 h		Significant mortality, total mortality at 0.1-0.001 concen-trations	Smith GR. 2001. Effects of acute exposure to a commercial formulation of glyphosate on the tadpoles of two species of anurans. B Environ Contam Tox 67: 483-488.

Pseudacris triseriata	Hylidae	Anura	Nearctic	Survivors from the first experiment at 0.0001 concentrations	Labor-atory	Kleeraway Grass & weed Killer RTU (ethoxylated tallowamine surfactant)	Development	0.0001 concen-tration		No renewal	336 h		No effects on development (final mass and Gosner-stage)	Smith GR. 2001. Effects of acute exposure to a commercial formulation of glyphosate on the tadpoles of two species of anurans. B Environ Contam Tox 67: 483-488.
Pseudacris triseriata	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup WeatherMAX	Survival, body mass and time to meta-morphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished meta-morphosis		No effects on survival and mass; marginally significant effects on time to meta-morphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.
Pseudacris triseriata	Hylidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	Survival, body mass and time to meta-morphosis	700 ppb (drinking water standard)		72 h	Until all survivors finished meta-morphosis		No effects on survival and mass; marginally significant effects on time to meta-morphosis	Williams BK, Semlitsch RD. 2010. Larval responses of three Midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Contam Toxicol 58: 819-827.

Rana cascadae	Ranidae	Anura	Nearctic	Early larvae (Gosner stage25)	Labor-atory	Roundup OriginalMAX	LC50	5.26 mg a.e./L	1.7 mg a.e./L	24 h	96 h		Anurans more sensitive than urodels	Relyea RA, Jones DK. 2009. The toxicity of Roundup OriginalMAX® to 13 species of larval amphibians. Environ Toxicol Chem 28: 2004-2008.
Rana cascadae	Ranidae	Anura	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	1.0 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; total mortality in the highest treatment after 24 h	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.
Rana cascadae	Ranidae	Anura	Nearctic	Larvae	Labor-atory	Roundup Original (POEA surfactant)	Survival, develop-ment, time to meta-morphosis, and behavior	2.0 mg a.e./L		168 h	1,032 h		Earlier meta-morphosis and reduced size	Cauble K, Wagner RS. 2005. Sublethal effects of the herbicide glyphosate on amphibian metamorphosis and development. B Environ Contam Tox 75: 429-435.
Rana luteiventris	Ranidae	Anura	Nearctic	Early larvae	Labor-atory	Roundup Regular (POEA surfactant)	LC50	3.8 mg a.e./L	0.7 mg a.e./L	No renewal	384 h		Significant different LC50 values among species, families, and orders; total mortality in the highest treatment after 24 h	King JJ, Wagner RS. 2010. Toxic effects of Roundup® Regular on Pacific Northwestern amphibians. Northwestern Nat 91: 318-324.

Rhinella arenarum	Bufonidae	Anura	Neo-tropic	Late larvae (Gosner stage36-38)	Labor-atory	Roundup UltraMAX	LC50, activity of 3 esterases and S-transferase	240 mg a.e./L	2.4 mg a.e./L	No renewal	48 h		Enzymatic inhibition, but significant different among herbicides just as acute toxiciy	Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC. 2011. Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. Arch Environ Contam Toxicol 60: 681-689.
Rhinella arenarum	Bufonidae	Anura	Neo-tropic	Late larvae (Gosner stage36-38)	Labor-atory	Infosato	LC50, activity of 3 esterases and S-transferase	240 mg a.e./L	38.8 mg a.e./L	No renewal	48 h		Enzymatic inhibition, but significant different among herbicides just as acute toxiciy	Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC. 2011. Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. Arch Environ Contam Toxicol 60: 681-689.

Rhinella arenarum	Bufonidae	Anura	Neo-tropic	Late larvae (Gosner stage36-38)	Labor-atory	Glifoglex	LC50, activity of 3 esterases and S-transferase	240 mg a.e./L	73.8 mg a.e./L	No renewal	48 h		Enzymatic inhibition, but significant different among herbicides just as acute toxiciy	Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC. 2011. Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. Arch Environ Contam Toxicol 60: 681-689.
Rhinella arenarum	Bufonidae	Anura	Neo-tropic	Late larvae (Gosner stage36-38)	Labor-atory	C-K Yuyos FAV	LC50, activity of 3 esterases and S-transferase	240 mg a.e./L	77.5 mg a.e./L	No renewal	48 h		Enzymatic inhibition, but significant different among herbicides just as acute toxiciy	Lajmanovich RC, Attademo AM, Peltzer PM, Junges CM, Cabagna MC. 2011. Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors. Arch Environ Contam Toxicol 60: 681-689.

Rhinella arenarum	Bufonidae	Anura	Neo-tropic	Adults	Labor-atory	Roundup Original (POEA surfactant)	Frequency of MNE after 5 d	600 mg a.e./L		48 h	120 h		No significant risk	Bosch B, Mañas F, Gorla N, Aiassa D. 2011. Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. J Tox Env Health Sci 3: 155-163.
Rhinella granulosa	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		7.2 mg a.e./L	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.

Rhinella granulosa	Bufonidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		6.5 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Rhinella granulosa	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		2.4 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		6.0 mg a.e./L	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		22.8 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		2.7 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		2.8 (mild), 5.1 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Agral 600 (60% NPE and unspecified concen-trations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		2.9 (mild), 5.4 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)			24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.5 (mild), 4.0 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.7 (mild), 4.4 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.3 (mild), 3.4 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.7 (mild), 4.2 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.5 (mild), 4.3 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25-30)	Labo-ratory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		3.5 (mild), 4.2 (full narcosis) mg/L NPE	24 h	96 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Late larvae (Gosner stage39-40)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		4.1 (mild), 4.1 (full narcosis) mg/L NPE	24 h	48 h	High temperature (30°C)	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)	4.0 mg/L NPE	3.6 (mild), 4.1 (full narcosis) mg/L NPE	No renewal	12 h	High temperature (30°C) and normal dissolved oxygen	High temp-eratures had little effect on EC50	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)	4.0 mg/L NPE	1.8 (mild), 2.2 (full narcosis) mg/L NPE	No renewal	12 h	High temperature (30°C) and low dissolved oxygen	High temperature-low dissolved oxygen resulted in a two to threefold increase in toxicity	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)	4.0 mg/L NPE	1.8 (full narcosis) mg/L NPE	No renewal	12 h	High temperature (30°C) and low dissolved oxygen	High temperature-low dissolved oxygen resulted in a two to threefold increase in toxicity	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Rhinella marina	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)	4.0 mg/L NPE	1.4 (full narcosis) mg/L NPE	No renewal	12 h	High temperature (30°C) and low dissolved oxygen	High temperature-low dissolved oxygen resulted in a two to threefold increase in toxicity	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Rhinella typhonius	Bufonidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		14.8 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Rhinella typhonius	Bufonidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		1.5 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Scinax nasicus	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage25-26)	Labor-atory	Glyfos (POEA surfactant)	LC50, development (mal-formations)	3.8 mg a.e./L	0.9 mg a.e./L	24 h	96 h		Mal-formations (especially of the gills) in all treatments, but increased with concen-tration	Lajmanovich RC, Sandoval MT, Peltzer PM. 2003. Induction of mortality and malformation in *Scinax nasicus* tadpoles exposed to glyphosate formulations. B Environ Contam Tox 70: 612-618.

Scinax ruber	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		6.9 mg a.e./L	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.
Scinax ruber	Hylidae	Anura	Neo-tropic	Juveniles	Meso-cosm	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		7.3 kg a.e./ha	No renewal	96 h	Layer of local soil	Differences between species; no risk	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval and juvenile Colombian frogs 2. Field and laboratory microcosm acute toxicity. J Toxicol Env Health A 72: 966-973.

Scinax ruber	Hylidae	Anura	Neo-tropic	Early larvae (Gosner stage 25)	Labor-atory	Glyphos (POEA surfactant) + Cosmo-Flux	LC50		1.6 mg a.e./L	24 h	96 h		Differences between species	Bernal MH, Solomon KR, Carrasquilla G. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. J Toxicol Env Health A 72: 961-965.
Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	10 mg a.e./L	2.0 mg a.e./L	96 h	48 h	Grassland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	10 mg a.e./L	2.0 mg a.e./L	96 h	216 h	Grassland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	Survival and weight	2.8 mg a.e./L		96 h	720 h	Grassland population	Significant effect on survival; no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	10 mg a.e./L	1.9 mg a.e./L	96 h	48 h	Cropland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	10 mg a.e./L	1.7 mg a.e./L	96 h	216 h	Cropland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea bombifrons	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	Survival and weight	2.8 mg a.e./L		96 h	720 h	Cropland population	Significant effect on survival; no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	7.5 mg a.e./L	2.3 mg a.e./L	96 h	48 h	Grassland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	7.5 mg a.e./L	1.9 mg a.e./L	96 h	216 h	Grassland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	Survival and weight	2.8 mg a.e./L		96 h	720 h	Grassland population	Significant effect on survival; no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	7.5 mg a.e./L	2.1 mg a.e./L	96 h	48 h	Cropland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	LC50	7.5 mg a.e./L	2.1 mg a.e./L	96 h	216 h	Cropland population	Significant effect, LC50 similar to EEC (2.8 mg a.e./L); no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Early larvae (Gosner stage 29-30)	Labor-atory	Roundup Weather-MAX	Survival and weight	2.8 mg a.e./L		96 h	720 h	Grassland population	Significant effect on survival; no significant differences between populations nor species	Dinehart SK, Smith LM, McMurry ST, Smith PN, Anderson TA, Haukos DA. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280 SL to larval Spea multiplicata and S. bombifrons from the Southern High Plains, USA. Environ Pollut 158: 2610-2617.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Super Concentrate	Survival	1.3 mg a.e./m²		No renewal	48 h	Moist paper towel	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Super Concentrate	Survival	1.3 mg a.e./m²		No renewal	48 h	Soil	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Ready-To-Use Plus	Survival	1.3 mg a.e./m²		No renewal	48 h	Moist paper towel	Significant effect	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup Weed and Grass Killer Ready-To-Use Plus	Survival	1.3 mg a.e./m²		No renewal	48 h	Soil	Significant effect	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup WeatherMAX	Survival	0.2 mg a.e./m²		No renewal	48 h	Moist paper towel	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.

Spea multiplicata	Scaphio-podidae	Anura	Nearctic	Meta-morphs	Labor-atory	Roundup WeatherMAX	Survival	0.2 mg a.e./m²		No renewal	48 h	Soil	No significant risk	Dinehart SK, Smith LM, McMurry ST, Anderson TA, Smith PN, Haukos DA. 2009. Toxicity of a glufosinate- and several glyphosate-based herbicides to juvenile amphibians from the Southern High Plains, USA. Sci Total Environ 407: 1065-1071.
Taricha granulosa	Salaman-dridae	Uro-dela	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	1250 mg a.e./L (intra-peritoneal)	No renewal	96 h		Low intraperitoneal toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.

Taricha granulosa	Salaman-dridae	Uro-dela	Nearctic	Adults	Labor-atory	Glyphosate isopropyl-amine salt	LD50; liver and kidney tissue damage	2700 mg/kg	> 2600 mg a.e./L (oral)	No renewal	96 h		Low intraperitoneal toxicity, very low oral toxicity; constant across species; no liver or kidney damage	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.
Taricha granulosa	Salaman-dridae	Uro-dela	Nearctic	Adults	Field	Glyphosate isopropyl-amine salt	Survival and distance moved	225 mg/kg		No renewal	168 h	Radio transmitter	No significant differences in average distance moved or mortality	McComb BC, Curtis L, Chambers CL, Newton M, Bentson K. 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ Sci Pollut R 15: 266-272.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	15.6 mg a.e./L (pH 6.0); 7.9 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Embryos more resistant; higher toxicity with higher pH level	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Early larvae (Gosner stage 25)	Labor-atory	Vision (POEA surfactant)	LC50	20 mg a.e./L	2.1 mg a.e./L (pH 6.0); 0.9 mg a.e./L (pH 7.0)	24 h	96 h	pH 4.5-8.5	Higher toxicity with higher pH level, if pH ≥ 7.5, LC50 at or below EEC (1.44 mg a.e./L)	Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23: 815-822.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Rodeo (without surfactant)	Survival (LC50), development (mal-formations)	Standard method FETAX	7296.8 mg a.e./L	24 h	96 h		Toxicity: POEA > Roundup > Rodeo; no significant malformation rate	Perkins PJ, Boermans HJ, Stephenson GR. 2000. Toxicity of glyphosate and triclopyr using the Frog Embryo Teratogenesis Assay-*Xenopus*. Environ Toxicol Chem 19: 940-945.
Xenopus laevis	Pipidae	Anura	Afrotropic	Embryos	Laboratory	Roundup Original (POEA surfactant)	Survival (LC50), development (malformations)	Standard method FETAX	9.3 mg a.e./L	24 h	96 h		Toxicity: POEA > Roundup > Rodeo; no significant malformation rate	Perkins PJ, Boermans HJ, Stephenson GR. 2000. Toxicity of glyphosate and triclopyr using the Frog Embryo Teratogenesis Assay-*Xenopus*. Environ Toxicol Chem 19: 940-945.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	POEA	Survival (LC50), development (mal-formations)	Standard method FETAX	6.8 mg a.e./L	24 h	96 h		Toxicity: POEA > Roundup > Rodeo; no significant malformation rate	Perkins PJ, Boermans HJ, Stephenson GR. 2000. Toxicity of glyphosate and triclopyr using the Frog Embryo Teratogenesis Assay-*Xenopus*. Environ Toxicol Chem 19: 940-945.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Melano-phore cells	Labor-atory	Roundup Original (POEA surfactant)	Effects on cell structure and transport	2 g/L		No renewal	24 h	Low/high pH	Cellular morphology and transport affected; uptake at high pH because POEA increased membrane permeability	Hedberg D, Wallin M. 2010. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. Toxicol In Vitro 24: 795-802.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Melano-phore cells	Labor-atory	Glyphosate isopropyl-amine salt	Effects on cell structure and transport	2 g/L		No renewal	24 h	Low/high pH	Cellular morphology and transport affected; no uptake at high pH	Hedberg D, Wallin M. 2010. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. Toxicol In Vitro 24: 795-802.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Melano-phore cells	Labor-atory	Gyphosate acid	Effects on cell structure and transport	2 g/L		No renewal	24 h	Low/high pH	Cellular morphology and transport affected; no uptake at high pH	Hedberg D, Wallin M. 2010. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. Toxicol In Vitro 24: 795-802.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Roundup Original (POEA surfactant)	Development (mal-formations)	430μM		No renewal	When controls reached desired stage		Roundup interferes with key molecular mechanisms regulating early development, leading to malformations	Paganelli A, Gnazzo V, Acosta H, Lopez SL, Carrasco AE. 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem Res Toxicol 23: 1586-1595.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Glyphosate acid	Development (mal-formations)	12 μM		No renewal	When controls reached desired stage		Glyphosate interferes with key molecular mechanisms regulating early development, leading to malformation s	Paganelli A, Gnazzo V, Acosta H, Lopez SL, Carrasco AE. 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem Res Toxicol 23: 1586-1595.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Late embryos (Nieuwkoo p & Faber stage 41)	Labor-atory	Roundup Original (POEA surfactant)	Survival and mal-formations	5 mg a.e./L		24 h	48 h		No mortality and no significant malformation rate	Lenkowski JR, Sanchez-Bravo G, McLaughlin KA. 2010. Low concentrations of atrazine, glyphosate, 2, 4-dichlorophenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis. J Environ Sci 22: 1305-1308.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Survival (LC50), malformation (EC50), terato-genicity indices (TI), and minimum concen-tration to inhibit growth (MCIG)	Standard method FETAX (8 mg/L NPE)	LC50 4.6, EC50 4.6, MCIG 3.0 mg/L NPE, TI 1.0	24 h	96 h		Concentration s between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmenta l malformation s or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Survival (LC50), malformation (EC50), terato-genicity indices (TI), and minimum concen-tration to inhibit growth (MCIG)	Standard method FETAX (8 mg/L NPE)	LC50 5.4, EC50 3.3, MCIG 1.0 mg/L NPE, TI 1.6	24 h	96 h		Concentration s between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmenta l malformation s or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Survival (LC50), malformation (EC50), terato-genicity indices (TI), and minimum concen-tration to inhibit growth (MCIG)	Standard method FETAX (8.2 mg/L NPE)	LC50 3.9, EC50 2.8, MCIG 2.0 mg/L NPE, TI 1.4	24 h	96 h		Concentration s between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmenta l malformation s or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Embryos	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Survival (LC50), malformation (EC50), terato-genicity indices (TI), and minimum concen-tration to inhibit growth (MCIG)	Standard method FETAX (10 mg/L NPE)	LC50 4.8 mg/L NPE	24 h	96 h		Concentrations between 1.0 and 10.0 mg/L will inhibit embryo growt andcause developmental malformations or cause mortality	Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat Toxicol 51: 19-29.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Early larvae (Gosner stage25)	Labor-atory	Teric GN8 (100% nonylphenol polyethoxy-late surfactant, NPE)	Narcosis (EC50)		1.1 (mild), 2.8 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.
Xenopus laevis	Pipidae	Anura	Afro-tropic	Early larvae (Gosner stage25)	Labor-atory	Agral 600 (60% NPE and unspecified concen-trations of oleic acid and 2-ethyl hexanol)	Narcosis (EC50)		1.2 (mild), 2.3 (full narcosis) mg/L NPE	24 h	48 h		EC50 values range from 1.1 (mild) to 12.1 (full narcosis) mg/L NPE	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Xenopus laevis	Pipidae	Anura	Afro-tropic	Early larvae (Gosner stage25)	Labor-atory	BS 1000 (100% alcohol alkoxylate)	Narcosis (EC50)			24 h	48 h		EC50 values range from 5.3 (mild) to 25.4 (full narcosis) mg/L alcohol alkoxylate	Mann RM, Bidwell JR. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and, two exotic frogs. Environ Pollut 114: 195-205.

Appendix B: Evaluation of the 'habitat exposure index' (HEI) to pesticides of each Annex II amphibian species.

Species	**Aquatic and terrestrial habitats (EF 1)** (literature-based)[a]	**Aquatic and terrestrial habitats (EF 1)** (logistic regressions)[b]	**Migration behavior (EF 2)**	**Breeding accumulations (EF 3)**	**HEI**	**References**
Caudata						
Golden-striped salamander (*Chioglossa lusitanica*)	(+)	+ (z = - 0.43, p > 0.05)	0	0	**1**	ARNTZEN 1981; OLIVEIRA 1997; ARNTZEN *et al.* 2009a; ORTIZ-SANTALIESTRA *et al.* 2010
* Golden alpine salamander (*Salamandra atra aurorae*)	0	NA [c]	0	0	**0**	GROSSENBACHER 1997a; ANDREONE *et al.* 2009a
Spectacled salamander *(Salamandrina terdigitata*)	+	NA [c]	0	0	**1**	VANNI & NISTRI 1997; SINDACO *et al.* 2009a
Italian crested newt (*Triturus carnifex*)	+	NA [c]	+	+	**3**	EDGAR & BIRD 2006; ROMANO *et al.* 2009
Northern crested newt (*T. cristatus*)	(+)	+ (z = - 1.22, p > 0.05)	+	+	**3**	ARNTZEN & BORKIN 1997; ARNTZEN *et al.* 2009b
Danube crested newt (*T. dobrogicus*)	+	NA [c]	+	+	**3**	ARNTZEN *et al.* 1997, 2009c

Southern crested newt (*T. karelinii*)	+	NA [c]	+	+	**3**	ARNTZEN *et al.* 2009d
Romanian smooth newt (*Lissotriton vulgaris ampelensis*)	++	NA [c]	+	+	**4**	ARNTZEN *et al.* 2009e
Carpathian newt (*L. montandoni*)	(+)	0 (z = - 3.61, p < 0.001)	+	+	**2**	ARNTZEN *et al.* 2009f
* Olm (*Proteus anguinus*)	+	NA [c]	0	0	**1**	DURAND 1997; ARNTZEN *et al.* 2009g
Ambrosi's cave salamander (*Hydromantes ambrosii*)	0	NA [c]	0	0	**0**	LANZA 1997a; ANDREONE *et al.* 2009b
Monte Albo cave salamander (*H. flavus*)	0	NA [c]	0	0	**0**	LANZA 1997b; LECIS *et al.* 2009
Sardinian cave salamander (*H. genei*)	(0)	+ (z = 1.44, p > 0.05)	0	0	**1**	LANZA 1997c; ANDREONE *et al.* 2009c
Imperial cave salamander (*H. imperialis*)	(0)	+ (z = 1.76, p > 0.05)	0	0	**1**	LANZA 1997d; ANDREONE *et al.* 2009d
North-west Italian cave salamander (*H. strinatii*)	(0)	0 (z = - 2.50, p < 0.05)	0	0	**0**	ANDREONE *et al.* 2009[e]

Supramonte cave salamander (*H. supramontis*)	0	NA [c]	0	0	**0**	LANZA 1997e; ANDREONE *et al.* 2009f
Anura						
* Mallorcan midwife toad (*Alytes muletensis*)	(0)	+ ($z = 0.13$, $p > 0.05$)	0	0	**1**	MARTINEZ RICA 1997; SERRA *et al.* 2009
Fire-bellied toad (*Bombina bombina*)	(++)	++ ($z = 7.57$, $p < 0.001$)	+	+	**4**	NÖLLERT & NÖLLERT 1992; GOLLMANN *et al.* 1997; AGASYAN *et al.* 2009a
Yellow-bellied toad (*B. variegata*)	(+)	0 ($z = - 3.05$, $p < 0.01$)	+	+	**2**	NÖLLERT, 1996; GREULICH & PFLUGMACHER 2004; KUZMIN *et al* 2009; WAGNER & FLOTTMANN 2011
Iberian painted frog (*Discoglossus galganoi*) including Spanish painted frog (*D. jeanneae*)	(++)	++ ($z = 3.05$, $p < 0.01$)	0	+	**3**	VEITH & MARTENS 1997a; BOSCH *et al.* 2009a,b
Corsican painted frog (*D. montalentii*)	0	NA [c]	0	0	**0**	VEITH & MARTENS 1997b; MIAUD *et al.* 2009
Tyrrhenian painted frog (*D. sardus*)	(+)	0 ($z = -3.86$, $p < 0.001$)	0	0	**0**	VEITH & MARTENS 1997c; ANDREONE *et al.* 2009g

Italian agile frog (*Rana latastei*)	+	NA [c]	+	+	**3**	GROSSENBACHER 1997b; TOCKNER *et al.* 2006; SINDACO *et al.* 2009b
* Common spadefoot (*Pelobates fuscus insubricus*)	++	NA [c]	+	+	**4**	NÖLLERT 1997; AGASYAN *et al.* 2009b

[a] For species without sufficient occurrence data for statistical analysis, the literature based ‘risk points’ were considered (n = 13)

[b] Only possible for 11 species

[c] NA = not sufficient occurrence data available in the databases ‘GBIF’, ‘HerpNET’ and ‘RACE’

* European priority species of the Habitats Directive

Appendix C: Justification for setting the 'Evaluation factors' (EF) 1 (habitat exposure risk), 2 (temporal coincidence of migration with pesticide applications) and 3 (breeding accumulations) for Annex II amphibians based on literature (in alphabetical order)

The Mallorcan midwife toad, *Alytes muletensis*, is restricted to the Sierra Tramuntana of northern Mallorca with only 500-1500 adult pairs (MARTÍNEZ RICA 1997). It can only be found near small streams in the local limestone mountains. Hence, there is a very low exposure risk to agrochemicals (EF 1 = 0) and other threats such as habitat destruction, introduced species, and emerging infectious diseases are important (SERRA *et al.* 2009). Furthermore, it moves short distances (EF 2 = 0) and shows no breeding accumulations (EF 3 = 0).

The Fire-bellied toad, *Bombina bombina*, occurs in a variety of wetland habitats, often within cultivated landscapes. It uses agricultural ponds and also drainage ditches for reproduction and dispersal (NÖLLERT & NÖLLERT 1992). Although floodplain destruction by river regulation is the main threat to the species and it is reported that it can occur in waters that have been polluted with industrial and agricultural chemicals in some regions, pollution of suitable wetland areas is considered to be a major threat to it (EF 1 = 2) (GOLLMANN *et al.* 1997; AGASYAN *et al.* 2009a). The species spends most of the year in its aquatic habitat (EF 2 = 1) but migrates to its nearby winter quarters such as stone piles that are also often situated within cultivated landscapes (EF 3 = 1). However, this semi-aquatic species can also hibernate in the mud on the bottom of water bodies and terrestrial migration mainly occurs during nighttime, which partly reduces its exposure risk to pesticides (NÖLLERT & NÖLLERT 1992).

The Yellow-bellied toad, *Bombina variegata*, inhabits a wide range of habitats (EF 1 = 1) including light forested landscapes, different wetland types such as floodplains and secondary habitats such as gravel pits. Although the species can tolerate slight water pollution and sometimes occurs even in highly polluted wetlands (KUZMIN *et al.* 2009), some pesticides are known to adversely affect this species (GREULICH & PFLUGMACHER 2004). It has also a semi-aquatic behavior like the Fire-bellied toad (EF 2 =1) and especially juveniles and young males can migrate longer distances (WAGNER & FLOTTMANN 2011). Hibernation takes place on land (NÖLLERT 1996) (EF 3 = 1).

Agriculture is increasing in its habitats and agrochemical pollution, mainly with insecticides, of the streams where the Golden-striped salamander, *Chioglossa lusitanica*, lives is supposed to be one of its major threats (ARNTZEN *et al.* 1981; OLIVEIRA 1997; ORTIZ-SANTALIESTRA *et al.* 2011). However, terrestrial habitats are mainly dense vegetation around forest streams, caves, and abandoned mines (ARNTZEN *et al.* 2009a) and there are not yet sufficient data to assess if this species is really in danger (OLIVEIRA 1997) (EF 1 = 1).The species only move short distances from the terrestrial habitats to the nearby reproduction streams (EF 2 = 0), and no breeding accumulations occur (ARNTZEN *et al.* 2009a) (EF 3 = 0).

The Iberian painted frog, *Discoglossus galganoi*, and the Spanish painted frog, *D. jeanneae*, are often present in cultivated areas and intensification of agriculture is supposed to be the main threat to the species (EF 1 = 2). They do not migrate long distances and are generally found within or in the direct vicinity to water (BOSCH *et al.* 2009a,b) (EF 2 = 0), but *D. galganoi* is present in its breeding sites (all kind of water bodies with preference to temporary ones) from October to late summer (EF 3 = 1), but overall little is known about the ecology of this species (NÖLLERT & NÖLLERT 1992; VEITH & MARTENS 1997a).

The Corsican painted frog, *Discoglossus montalentii,* is endemic to Corsica and is strongly associated with running waters in high-altitude pristine woods and forests and is absent from coastal lowlands (VEITH & MARTENS 1997b; MIAUD *et al.* 2009) (EF 1 = 0). The eggs are deposited under rocks and stones in mountain streams, with larvae developing in these streams; little is further known about the ecology of this species (VEITH & MARTENS 1997b; MIAUD *et al.* 2009) (EF 2 + 3 = 0).

The Tyrrhenian painted frog, *Discoglossus sardus*, inhabits a variety of habitats including cultivated areas, but it is apparently not seriously threatened by human activities except for damming of streams and increased water abstraction for tourism (ANDREONE *et al.* 2009g) (EF 1 = 1). Little is known about the ecology of this species (VEITH & MARTENS 1997c) (EF 2 + 3 = 0).

All *Hydromantes* species (cave salamanders) are more or less unlikely at risk to pesticide exposure because they live in remote areas (caves and forest floors in the mountains) (LANZA 1997a,b,c,d,e) (EF 1 = 0). They are independent from open water for reproduction since they are fully terrestrial, move short distances (EF 2 = 0), including direct development of eggs laid under stones or leaf litter or in crevices (BÖHME *et al.* 1999; CHIARI *et al.* 2012) (EF 3 = 0).

Lissotriton newt species occur in a wide range of habitats, from forested to cultivated landscapes, and use also agricultural ponds, irrigation channels, etc. *Lissotriton montandoni* can also be present in modified habitats, but in its range pollution by domestic sewage seems to be a higher threat than agrochemicals (ARNTZEN *et al.* 2009f). *Lissotriton vulgaris ampelensis* lives in the northern part of the Romanian Plateau (NÖLLERT & NÖLLERT 1992). Because agriculture in general is becoming strongly intensified in Romania today (BALTEANU & POPOVICI 2010), impacts on this subspecies can be assumed (EF 1 = 2 for *L. v. ampelensis*, EF 1 =1 for *L. montandoni*). These newts migrate between winter, breeding, and summer habitats (EF 2 = 1) and are present in their breeding ponds for several months (EF 3 = 1).

The Common spadefoot, *Pelobates fuscus*, is among the amphibian species with the highest adaption to agricultural land. This also holds for the subspecies *P. f. insubricus* that only occur in a limited range of the Po Valley in Italy (NÖLLERT 1997) and constitutes a highly threatened lineage with strong recent declines (AGASYAN *et al.* 2009b). Common spadefoots have their aquatic and terrestrial habitats often directly within agricultural landscapes and since the species is threatened by intensification of agriculture *s.l.* (*e.g.* ploughing) including pollution with agrochemicals (eutrophication of breeding ponds etc.), this can also be assumed for the Italian subspecies (NÖLLERT 1997) (EF 1 = 2). The species migrates between winter, breeding, and summer habitats (EF 2 = 1) and shows breeding accumulations in late spring (EF 3 = 1).

The Olm, *Proteus anguinus*, is highly dependent on clean water and, therefore, very susceptible to pollution. Despite its stygobiotic lifetype (EF 2 + 3 = 0), main threats to *P. anguinus* are changes of the land use above its subterranean habitat, which may lead to increasing water pollution (ARNTZEN *et al.* 2009b). Among others water pollution is a main threat to the species and it is therefore seen to be endangered (DURAND 1997) (EF 1 = 1).

Primary habitats of the Italian agile frog, *Rana latastei*, are semi-hygrophilous forest (Querco-Carpinetum boreoitalicum) along streams and other parts of natural floodplains (GROSSENBACHER 1997b; TOCKNER *et al.* 2006). Today, it can also occur in agricultural irrigation ditches, but only if these are close to forest remnants for over-wintering (EF 1 = 1). The species conducts annual migrations (EF 2 = 1) and forms breeding associations (EF 3 = 1) like other European brown frogs (GROSSENBACHER 1997b; SINDACO *et al* 2009b). Besides habitat destruction, water pollution is seen to be a main threat of the species (GROSSSENBACHER 1997b).

The Golden alpine salamander, *Salamandra atra aurorae*, is largely restricted to the forested plateau in Bosco del Dosso and Val Rensola in north-east Italy, with no direct threat due to agricultural practices EF 1 = 0). There exists no information about migration activities leading the species close to agricultural land (EF 2 = 0). The species is not associated with open water because of its viviparous mode of reproduction (ANDREONE *et al.* 2009a) (EF 3 = 0). However, it is highly endangered due to its very small range of less than 50 km² (GROSSENBACHER 1997a).

The Spectacled salamander, *Salamandrina terdigitata*, as a typical hill species, mainly occurs in non-modified habitats like forests with clear cold streams or oligotrophic ponds as breeding sites, and only occasionally it uses agricultural ponds such as drinking troughs for reproduction (VANNI & NISTRI 1997; SINDACO *et al.* 2009a). Nevertheless, SINDACO *et al.* (2009a) and VANNI & NISTRI (1997) stated localized declines through aquatic pollution (EF 1 = 1). It does not move long distances and only females of this species are aquatic during the short oviposition phase (SINDACO *et al.* 2009a) (EF 2 + 3 = 0).

Habitats of the Northern crested newt, *Triturus cristatus*, are among others situated within cultivated landscapes (NÖLLERT & NÖLLERT 1992; BÖHME *et al.* 1999). It also uses a wide range of small water bodies for reproduction, but changes in water quality due to agrochemical pollution is supposed to be a major threat to the species (ARNTZEN *et al.* 2009b) and it especially suffers from modernization of farming methods (ARNTZEN & BORKIN 1997) (EF 1 = 1). It migrates between winter, breeding and summer habitats (NÖLLERT & NÖLLERT 1992; BÖHME *et al.* 1999) (EF 2 = 1). Furthermore, the species shows a prolonged breeding time and is present in its aquatic habitats for several months (EF 3 = 1). The same holds true for other crested newt species. Pollution of wetlands by agrochemicals appear to be the main threats to *T. carnifex* (ROMANO *et al.* 2009), *T. dobrogicus* (ARNTZEN *et al.* 2009c), and *T. karelinii* (ARNTZEN *et al.* 2009d). These species scores the same risk points for all EF.

Appendix D: 'Land use with regular pesticide applications' (LPA) within 'special areas of conservation' (SAC) that were created for Annex II amphibian species, within 1 km buffer around available presence data, 'habitat exposure index' (HEI), and 'Pesticide risk factors' (PRF) for each species, sorted after total size of SAC. Above-average PRF are written in bold.

Species	**No. of SAC**[a]	**Area (km²) of SAC (Ø ± SE)**	**LPA**[b] **(km²) within SAC (proportion; Ø ± SE)**	**Proportion (± SE) of LPA**[b] **within buffers**	**Habitat exposure index (HEI)**	**Pesticide risk factor (PRF)**
Bombina variegata	1,463	118,124.61	15,336.25	21.88 ± 3.65	2	0.07
		(80.74 ± 5.6)	(12.98%; 10.48 ± 1.1)	(n = 100)		
Triturus cristatus	2,086	98,903.09	17,121.24	27.22 ± 3.15	3	**0.13**
		(47.14 ± 3.34)	(17.31 %; 8.16 ± 0.79)	(n = 100)		
Bombina bombina	1,256	82,281.94	17,780.90	64.16 ± 3.01	4	**0.22**
		(65.51 ± 5.45)	(21.61 %; 14.16 ± 1,28)	(n = 100)		
Discoglossus galganoi	266	67,873.21	16,870.72	32.57 ± 3.45	3	**0.19**
(including *D. jeanneae*)		(255.16 ± 24.20)	(24.86 %; 63.42 ± 8.54)	(n = 100)		
Triturus carnifex	585	32,525.88	6,120.53	NA [c]	3	**0.14**
		(55.46 ± 5.44)	(18.82 %; 10.46 ± 1.26)			
Triturus karelinii	156	31,068.72	5,941.67	NA [c]	3	**0.14**
		(191.52 ± 31.84)	(19.12 %; 37.46 ± 6.48)			

Lissotriton montandoni	123	20,727.22 (168.51 ± 28.83)	1,100.73 (5.31 %; 8.95 ± 2.70)	20.59 ± 3.61 (n = 39)	2	0.03
Salamandrina terdigitata	210	15,869.13 (75.57 ± 11.19)	1,724.77 (10.87 %; 8.21 ± 1.78)	NA [c]	1	0.03
Triturus dobrogicus	130	14,884.13 (114.49 ± 35.56)	3,382.61 (22.73 %; 26.02 ± 4.91)	NA [c]	3	**0.17**
Chioglossa lusitanica	98	13,130.23 (133.98 ± 20.52)	1,488.16 (11.33 %; 15.19 ± 3.41)	32.57 ± 2.70 (n = 100)	1	0.03
Discoglossus sardus	81	6,752.40 (83.36 ± 12.77)	914.20 (13.54 %; 11.29 ± 3.71)	8.54 ± 2.38 (n = 59)	0	0.00
Rana latastei	176	3,883.97 (22.07 ± 4.69)	1,234.56 (31.79 %; 7.01 ± 1.83)	NA [c]	3	**0.24**
Proteus anguinus	28	3,084.15 (110.15 ± 45.29)	270.16 (8.76 %; 9.65 ± 3.97)	NA [c]	1	0.02
Lissotriton vulgaris ampelensis	13	2,917.38 (224.41 ± 66.07)	173.73 (5.95 %; 13.36 ± 4.41)	NA [c]	4	0.06
Hydromantes strinatii	59	2,687.54 (45.55 ± 12.62)	29.74 (1.11 %; 0.50 ± 0.09)	7.94 ± 2.43 (n = 19)	0	0

Pelobates fuscus insubricus	27	1,286.52 (46.98 ± 17.42)	507.97 (40.04 %; 18.81 ± 10.39)	NA [c]	4	**0.40**
Hydromantes imperialis	5	979.48 (195.90 ± 95.40)	29.82 (3.04 %; 5.96 ± 3.00)	23.13 ± 5.83 (n = 11)	1	0.01
Discoglossus montalentii	14	771.39 (55.10 ± 17.50)	5.59 (0.73 %; 0.40 ± 0.25)	NA [c]	0	0
Hydromantes genei	4	740.19 (185.05 ± 51.53)	34.12 (4.61 %; 8.53 ± 0.46)	25.45 ± 7.04 (n = 16)	1	0.01
Hydromantes supramontis	2	524.84 (262.42 ± 27.52)	15.86 (3.02 %; 7.93 ± 5.22)	NA [c]	0	0
Salamandra atra aurorae	2	288.59 (144.29 ± 5.57)	2.09 (0.72 %; 1.05 ± 1.05)	NA [c]	0	0
Alytes muletensis	6	196.79 (32.80 ± 15.05)	3.87 (1.97 %; 0.65 ± 0.30)	17.93 ± 4.49 (n = 28)	1	0.01
Hydromantes ambrosii	7	91.67 (13.10 ± 3.77)	3.85 (4.20 %; 0.55 ± 0.52)	NA [c]	0	0
Hydromantes flavus	1	88.50	6.51 (7.36 %)	NA [c]	0	0

Ø 0.08 ± 0.01

[a] Excluding Greece and the UK due to the lack of land cover data; therefore, also *Lyciasalamandra luschani* that only occurs in Greek SAC could not been assessed.

[b] LPA = Land use with regular pesticide applications according to its CORINE land cover classes

[c] NA = not sufficient occurrence data available in the databases ‘GBIF’, ‘HerpNET’, and ‘RACE’

Appendix D: Differences in 'land use with regular pesticide applications' (LPA) within national 'special areas of conservation' (SAC) that were created for Annex II amphibian species, which occur in more than one EU member state (n = 11), and 'national pesticide risk factors' (NPRF) for each species, sorted after total size of SAC. Statistically significant national differences and above-average NPRF are written in bold.

Species, national differences[a]	**Country**	**No. of national SAC[b]**	**Total area (km^2) of national SAC (Ø ± SE)**	**LPA[c] (km^2) within all national SAC (proportion; Ø ± SE)**	**Average proportion of LPA[c] (km^2) within national SAC**	**National pesticide risk factor (NPRF) [d]**
Bombina variegata F = 44.14, df = 1, **p < 0.001**	Austria	75	5,654.71 (75.40 ± 18.31)	570.72 (10.9 %; 7.61 ± 2.09)	15.59 ± 2.66	0.05
	Bulgaria	110	25,899.58 (235.45 ± 43.70)	4,405.33 (17.01 %; 40.05 ± 8.25)	29.23 ± 2.51	0.09
	Czech Republic	20	1,218.44 (71.67 ± 63.41)	201.67 (16.55 %; 11.86 ± 10.36)	42.65 ± 9.24	0.08
	Germany	423	8,535.53 (20.18 ± 1.83)	918.80 (10.76 %; 2.17 ± 0.17)	19.69 ± 1.17	0.05
	France	145	7,362.24 (50.77 ± 8.78)	1,094.27 (14.86 %; 7.55 ± 1.74)	15.22 ± 1.37	0.07
	Hungary	30	3,949.80 (131.66 ± 41.27)	675.70 (17.11 %; 22.52 ± 14.53)	10.75 ± 3.54	0.09
	Italy	366	25,734.98 (70.31 ± 8.34)	4,388.28 (17.05 %; 11.99 ± 2.69)	22.91 ± 1.57	0.09

	Luxembourg	2	9.33	0.36	4.88 ± 2.54	0.02
			(4.67 ± 1.94)	(3.83 %; 0.18 ± 0.02)		
	Netherlands	2	26.43	5.33	34.30 ± 16.23	0.10
			(13.22 ± 11.50)	(20.17 %; 2.67 ± 1.80)		
	Poland	44	4,565.43	395.93	20.30 ± 3.92	0.04
			(103.76 ± 31.64)	(8.67 %; 9.00 ± 2.81)		
	Romania	84	20,037.79	1,262.94	8.89 ± 1.75	0.03
			(238.55 ± 40.72)	(6.30 %; 15.03 ± 3.61)		
	Slovenia	10	1,500.54	461.07	39.30 ± 9.16	**0.15**
			(150.05 ± 57.53)	(30.73 %; 46.11 ± 23.87)		
	Slovakia	152	13,629.04	955.85	14.93 ± 2.15	0.04
			(89.66 ± 16.81)	(7.01 %; 6.29 ± 1.96)		
Triturus cristatus [e]	Austria	15	678.06	159.41	14.01 ± 5.58	**0.18**
$F = 68.09$, $df = 1$, $\mathbf{p < 0.001}$			(45.20 ± 15.24)	(23.51 %; 10.63 ± 4.47)		
	Belgium	68	1,084.17	274.90	27.80 ± 2.82	**0.19**
			(15.94 ± 2.20)	(25.36 %; 4.04 ± 0.73)		
	Bulgaria	4	2,581.31	430.88	12.23 ± 3.66	0.13
			(645.33 ± 522.41)	(16.69 %; 107.72 ± 94.52)		

Czech Republic	63	747.48 (11.86 ± 5.68)	119.49 (15.99 %; 1.90 ± 0.89)	38.25 ± 5.18	0.12
Germany	1,072	16,937.34 (15.80 ± 0.97)	2,467.37 (14.57 %; 2.30 ± 0.14)	23.94 ± 0.83	0.11
Denmark	70	4,016.55 (55.02 ± 12.06)	456.25 (11.36 %; 6.25 ± 1.37)	20.94 ± 2.59	0.09
Estonia	13	477.63 (36.47 ± 20.49)	157.66 (33.01 %; 12.13 ± 7.64)	43.97 ± 10.46	**0.25**
Finland	6	14.84 (2.47 ± 0.90)	3.97 (26.73 %; 0.66 ± 0.56)	15.19 ± 10.83	**0.20**
France	177	12,983.17 (74.88 ± 21.14)	2,675.79 (20.61 %; 15.59 ± 6.39)	12.96 ± 1.09	**0.15**
Hungary	80	9,238.31 (115.48 ± 21.54)	2,717.56 (29.42 %; 33.97 ± 7.49)	25.68 ± 2.18	**0.22**
Lithuania	8	115.09 (18.85 ± 8.55)	12.67 (11.01 %; 2.11 ± 1.49)	8.40 ± 3.48	0.08
Luxembourg	19	250.20 (13.17 ± 3.94)	44.19 (17.66 %; 2.33 ± 1.00)	18.68 ± 4.34	0.13
Latvia	20	3,260.14	763.06	22.00 ± 4.58	**0.18**

			(163.01 ± 49.89)	(23.41 %; 38.15 ± 16.84)		
	Netherlands	37	1,484.02 (40.11 ± 24.47)	111.79 (7.53 %; 3.02 ± 0.98)	14.42 ± 2.30	0.06
	Poland	240	26,929.89 (112.21 ± 12.15)	4,920.75 (18.27 %; 20.50 ± 2.89)	18.97 ± 1.25	**0.14**
	Romania	51	12,541.42 (245.91 ± 55.76)	1,051.42 (8.38 %; 20.62 ± 5.63)	11.09 ± 2.65	0.06
	Sweden	133	1,215.49 (9.14 ± 3.78)	69.35 (5.71 %; 0.52 ± 0.26)	16.24 ± 2.62	0.04
	Slovakia	19	4,347.99 (228.84 ± 67.21)	684.74 (15.75 %; 36.04 ± 15.79)	23.78 ± 6.66	0.12
Bombina bombina $F = 59.53$, $df = 1$, $\boldsymbol{p < 0.001}$	Austria	18	1,590.36 (88.35 ± 28.47)	471.08 (29.62 %; 26.17 ± 9.84)	34.48 ± 5.85	**0.30**
	Bulgaria	114	10,865.55 (95.31 ± 18.36)	3,634.19 (33.45 %; 31.88 ± 7.02)	40.57 ± 2.59	**0.34**
	Czech Republic	88	858.93 (9.76 ± 4.31)	157.21 (18.30 %; 1.79 ± 0.67)	53.22 ± 4.33	**0.18**
	Germany	342	5,272.35 (15.42 ± 1.44)	1,075.14 (20.39 %; 3.14 ± 0.28)	29.80 ± 1.53	**0.20**

Denmark	7	1,295.96 (185.14 ± 72.63)	121.27 (9.36 %; 17.32 ± 8.29)	19.69 ± 7.10	0.09
Hungary	258	18,556.09 (71.10 ± 8.91)	4,272.33 (23.02 %; 16.37 ± 3.44)	19.57 ± 1.16	**0.23**
Lithuania	8	348.51 (49.78 ± 22.93)	24.31 (6.97 %; 3.47 ± 2.27)	8.13 ± 2.93	0.07
Latvia	2	38.27 (19.14 ± 19.12)	4.53 (11.84 %; 2.27 ± 2.25)	55.90 ± 44.10	0.12
Poland	295	29,202.50 (98.98 ± 10.83)	5,466 (18.72 %; 18.55 ± 2.54)	19.37 ± 1.13	**0.19**
Romania	34	11,148.56 (337.61 ± 136.96)	1,699.97 (15.25 %; 51.45 ± 14.82)	19.17 ± 2.90	**0.15**
Sweden	6	4.52 (0.75 ± 0.23)	0.72 (15.89 %; 0.12 ± 0.03)	36.47 ± 16.50	**0.16**
Slovenia	4	114.18 (28.55 ± 19.11)	37.85 (33.15 %; 9.46 ± 6.91)	26.98 ± 3.59	**0.33**
Slovakia	79	3,427.86 (43.95 ± 15.95)	933.58 (27.24 %; 11.97 ± 5.21)	27.36 ± 3.73	**0.27**

Triturus carnifex	Austria	25	2,235.59	253.31	22.25 ± 4.48	0.08
$F = 0.78$, $df = 1$, $p > 0.05$			(89.42 ± 34.87)	(11.33 %; 10.13 ± 3.31)		
	Bulgaria	1	688.50	84.40	12.26	0.09
				(12.26 %)		
	Czech Republic	6	0.35	0.27	58.45 ± 19.35	**0.57**
			(0.06 ± 0.02)	(76.37 %; 0.04 ± 0.02)		
	Hungary	3	913.35	236.73	18.89 ± 19.35	**0.19**
			(304.45 ± 144.96)	(25.92 %; 78.91 ± 39.15)		
	Italy	538	27,013.62	5,052.98	32.70 ± 1.44	**0.14**
			(50.21 ± 5.33)	(18.71 %; 9.39 ± 1.23)		
	Slovenia	12	1,674.46	492.84	36.49 ± 7.97	**0.22**
			(139.54 ± 48.88)	(29.43 %; 41.07 ± 20.07)		
Lissotriton montandoni	Czech Republic	5	1,230.74	196.96	21.92 ± 17.97	0.12
$F = 0.08$, $df = 1$, $p > 0.05$			(246.15 ± 239.50)	(16.00 %; 39.39 ± 39.34)		
	Poland	29	4,424.28	363.07	12.44 ± 3.53	0.06
			(152.56 ± 45.60)	(8.21 %; 12.52 ± 4.12)		
	Romania	32	7,598.90	101.76	1.01 ± 0.43	0.01
			(237.47 ± 78.33)	(1.34 %; 3.18 ± 1.83)		

	Slovakia	57	7,473.29	438.95	12.23 ± 3.17	0.04
			(131.11 ± 32.05)	(5.87 %; 7.70 ± 4.17)		
Triturus dobrogicus	Austria	12	790.37	210.84	34.32 ± 6.18	**0.20**
$F = 14.87$, $df = 1$, $\boldsymbol{p < 0.01}$			(65.86 ± 17.21)	(26.68 %; 17.57 ± 3.80)		
	Bulgaria	41	2,878.10	460.64	36.28 ± 4.15	0.12
			(70.20 ± 18.66)	(16.00 %; 26.18 ± 6.24)		
	Czech Republic	2	97.95	17.13	22.69 ± 5.28	0.13
			(48.98 ± 48.17)	(17.49 %; 8.57 ± 8.34)		
	Hungary	41	3,934.86	656.10	14.82 ± 1.88	0.13
			(95.97 ± 17.83)	(16.67 %; 18.58 ± 6.08)		
	Romania	12	6,150.48	910.96	21.74 ± 5.10	0.11
			(512 ± 366.43)	(14.81 %; 75.91 ± 36.83)		
	Slovakia	22	1,032.37	408.50	13.93 ± 3.51	**0.30**
			(46.93 ± 21.55)	(39.57 %; 18.57 ± 11.72)		
Chioglossa lusitanica	Spain	80	8,493.75	717.89	13.27 ± 1.42	0.02
$F = 1.64$, $df = 1$, $p > 0.05$			(106.17 ± 18.43)	(8.45 %; 8.97 ± 2.91)		
	Portugal	18	4,636.48	770.26	19.89 ± 3.78	0.04
			(257.58 ± 70.47)	(16.61 %; 42.79 ± 11.41)		

Discoglossus sardus	France	37	2,291.42	20.14	7.52 ± 2.35	0.00
F = 15.45, *df* = 1, ***p* < 0.001**			(61.93 ± 20.16)	(0.88 %; 0.54 ± 0.16)		
	Italy	44	4,460.98	894.06	19.81 ± 3.76	0.00
			(101.39 ± 15.99)	(20.04 %; 20.32 ± 6.56)		
Rana latastei	Italy	174	3,806.61	1,211.66	49.03 ± 2.69	**0.24**
F = 0.08, *df* = 1, p > 0.05			(21.88 ± 4.74)	(31.83 %; 6.96 ± 1.85)		
	Slovenia	2	77.36	22.90	49.07 ± 31.32	**0.22**
			(38.68 ± 24.04)	(29.60 %; 11.45 ± 0.32)		
Proteus anguinus	Italy	2	281.49	15.40	35.82 ± 0.84	0.02
F = 1.28, *df* = 1, *p* > 0.05			(109.25 ± 12.72)	(7.05 %; 7.70 ± 1.80)		
	Slovenia	27	2,865.66	254.76	38.04 ± 6.88	0.02
			(110.22 ± 48.84)	(8.89 %; 9.80 ± 4.28)		
Hydromantes strinatii	France	14	1,156.39	7.47	2.98 ± 1.24	0.00
F = 0.00, *df* = 1, *p* > 0.05			(82.60 ± 46.95)	(0.65 %; 0.53 ± 0.22)		
	Italy	44	1,513.80	21.49	2.70 ± 0.68	0.00
			(34.40 ± 7.92)	(1.42 %; 0.49 ± 0.10)		
						Ø 0.13 ± 0.01

[a] Differences between group means of LPA proportions in national SAC were checked using one-way ANOVA; some data had to be Boxcox-transformed prior analysis

[b] Excluding Greece due to the lack of land cover data; therefore, also *Lyciasalamandra luschani* that only occurs in Greek SAC could not been evaluated.

[c] LPA = Land use with regular pesticide applications according to its CORINE land cover classes (for details see chapter 2.1.).

[d] = calculated with proportion of LPA within all national SAC

[e] = SAC, which were created for *T. cristatus* in the UK have not been evaluated due to lack of actual land cover data

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Appendix E: Contingency table that contains the results of the Bonferroni-corrected post-hoc-tests for *Bombina variegata*, *Triturus cristatus*, *Bombina bombina* and *Triturus dobrogicus*. Post-hoc-tests for other species did not show significant differences between member states. n.s. = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001

AT = Austria; BE = Belgium; BG = Bulgaria; CZ = Czech Republic; DE = Germany; DK = Denmark; EE = Estonia; FI = Finland; FR = France; HU = Hungary; IT = Italy; LU =Luxembourg; LT = Lithuania; LV = Latvia; NL = Netherlands; PL = Poland; RO = Romania; SE = Sweden; SI = Slovenia; SK = Slovakia

Bombina variegata

	AT	**BG**	**CZ**	**DE**	**FR**	**HU**	**IT**	**LU**	**NL**	**PL**	**RO**	**SI**	**SK**
AT		*	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BG	*		n.s.	*	**	*	n.s.	n.s.	n.s.	n.s.	***	n.s.	***
CZ	**.	n.s.		**	***	**	n.s.	n.s.	n.s.	n.s.	***	n.s.	***
DE	n.s.	*	**		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.
FR	n.s.	**	***	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
HU	n.s.	*	**	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	***	n.s.	n.s.
LU	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.
NL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.
PL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.

RO	n.s.	***	***	***	n.s.	n.s.	***	n.s.	n.s.	n.s.		*	n.s.
SI	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*		n.s.
SK	n.s.	***.	***.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Triturus cristatus

	AT	**BE**	**BG**	**CZ**	**DE**	**DK**	**EE**	**FI**	**FR**	**HU**	**LU**	**LT**	**LV**	**NL**	**PL**	**RO**	**SE**	**SK**
AT		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BE	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BG	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CZ	n.s.	n.s.	n.s.		**	*	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	***	***	***	***	n.s.
DE	n.s.	n.s.	n.s.	**		n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DK	n.s.	n.s.	n.s.	*	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
EE	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	**	n.s.	n.s.	n.s.	n.s.	*	n.s.	**	*	n.s.
FI	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FR	n.s.	**	n.s.	***	***	n.s.	**	n.s.		*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
HU	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LU	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

LT	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LV	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.
NL	n.s.	n.s.	n.s.	***	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.
PL	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
RO	n.s.	n.s.	n.s.	***	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.
SE	n.s.	n.s.	n.s.	***	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.
SK	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Bombina bombina

	AT	**BG**	**CZ**	**DE**	**DK**	**HU**	**LT**	**LV**	**PL**	**RO**	**SE**	**SI**	**SK**
AT		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BG	n.s.		*	**	n.s.	***	*	n.s.	***	**	n.s.	n.s.	*
CZ	n.s.	*		***	n.s.	***	***	n.s.	***	***	n.s.	n.s.	***
DE	n.s.	**	***		n.s.	***	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.
DK	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
HU	n.s.	***	***	***	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LT	n.s.	*	***	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

LV	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.
PL	n.s.	***	***	***	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.
RO	n.s.	**	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.
SE	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.
SI	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.
SK	n.s.	*	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Triturus dobrogicus

	AT	**BG**	**CZ**	**HU**	**RO**	**SK**
AT		n.s.	n.s.	*	n.s.	n.s.
BG	n.s.		n.s.	***	n.s.	***
CZ	n.s.	n.s.		n.s.	n.s.	n.s.
HU	*	***	n.s.		n.s.	n.s.
RO	n.s.	n.s.	n.s.	n.s.		n.s.
SK	n.s.	***	n.s.	n.s.	n.s.	

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Erklärung

Hiermit versichere ich, dass mir die derzeitige Promotionsordnung bekannt ist und dass ich die vorliegende Dissertation selbständig verfasst habe. Ich habe für die Arbeit keine anderen als die angegebenen Quellen und Hilfsmittel genutzt und die Ergebnisse anderer Beteiligter sowie inhaltlich und wörtlich aus anderen Werken entnommene Stellen und Zitate wurden als solche kenntlich gemacht. Die Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen oder wurde von dieser als Teil einer Prüfungsleistung angenommen.

Trier, Juni 2015

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