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Possible correlation of the worldwide amphibian decline and the increasing use of glyphosate in the agrarian industry

BfN-Skripten 343

2013

Federal Agency for Nature Conservation

Trier University

Biogeographie

Expert opinion on the

Possible correlation of the worldwide amphibian decline and the increasing use of glyphosate in the agrarian industry

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Cover picture: The picture shows a pair of Common frogs (*Rana temporaria*) and a Common spadefoot toad (*Pelobates fuscus*). Both anurans regularly occur within cultivated landscapes and are, therefore, at potential risk of exposure to agrochemicals. The pond on the picture is situated within an agrarian landscape near Münster, Germany, and constitutes a breeding site of a large population of Northern crested newts (*Triturus cristatus*) (All photos taken by U. Schulte).

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This publication is included in the literature database “***DNL-online***” (www.dnl-online.de)

BfN-Skripten are not available in book trade but can be downloaded in a pdf version from the internet at: http://www.bfn.de/0502_skripten.html

Publisher: Bundesamt für Naturschutz (BfN)
Federal Agency for Nature Conservation
Konstantinstrasse 110
53179 Bonn, Germany
URL: http://www.bfn.de

Printed by the printing office of the Federal Ministry of Environment, Nature Conservation and Nuclear Safety.

Printed on 100% recycled paper.

ISBN 978-3-89624-078-1

Bonn, Germany 2013

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I. SUMMARY

Background

Worldwide amphibian populations are showing dramatic, non-natural negative trends and even species extinctions are documented. Scientists have long debated the causes of these declines. Multiple factors, including their interactions, are probably at work. Environmental contamination, e.g. due to pesticide applications, is discussed to be one reason. In this context, we ask which impact had, already has or will have the on-going change from conventional farming to agriculture with genetically modified (GM) crops on amphibians. This includes changes in weed management (to the exclusive use of non-selective herbicides) but also expansion and aggregation of agricultural areas. In the Americas, GM crops are already dominating some crop cultivations. For example, the total amount of GM crops in soy and cotton cultivations already represents 81%. In Europe, especially in Germany, GM crop cultivation is still standing at the beginning. This expert opinion mainly deals with the different herbicide application practices in conventional farming and farming with GM crops and possible impacts on amphibians. The majority of GM crops are herbicide-resistant (HR), i.e. they were genetically engineered to resist the use of non-selective (also called broad-spectrum) herbicides for weed control. Hence, an increasing cultivation of GM crops went along with an increasing application of non-selective herbicides, usually with glyphosate (GLY) as the active ingredient. Conversely, cultivation of HR crops should replace or at least minimise applications of selective, 'conventional' herbicides and less herbicide applications should be necessary, so that finally cumulative use of herbicides should decrease. Furthermore, the environmental safety of GLY – compared to other herbicides – is often highlighted. Although these benefits of GM crop cultivation could be observed for the first couple of years, when HR crop cultivation started, practical experience in the Americas has shown that the system consisting of HR crop and complementary non-selective herbicide often leads to a renunciation of crop rotation and a strong reliance on GLY as only weed control measurement. There seems to be especially no rotation of traits, so that consecutively glyphosate-based herbicides (GBH) have been regularly applied on the same fields. Hence GLY-resistant weeds have developed (and are reported) worldwide. These GLY-resistant weeds were combated with more non-selective herbicide or even a re-use of selective herbicides. In the long-term, the goal of decreasing herbicide use apparently could not be reached (although weed resistances are not a specific problem of HR crops but of an unsustainable farming). With regard to the environmental safety of GLY it should be kept in mind that the applied GBH include added substances, especially surfactants, which are usually more toxic than the active ingredient itself.

We here reviewed literature and databases on the basis of following key questions:

(i) Which concentrations of GLY and its main metabolite aminomethylphosphonic acid (AMPA) can be found in the environment?

(ii) What do we actually know about the impacts of GLY and its formulations on amphibians?

(iii) Can there be identified effects on amphibians as a result of the use of GLY coupled with biotic or abiotic stressors?

(iv) What are possible exposure pathways to different amphibian life-stages?

(v) Are amphibians differently affected by the cultivation of conventional crops compared with the HR crops especially with respect to different weed management systems?

Furthermore, we conducted a statistical, macroecological approach concerning the questions:

(vi) Do the agricultural change in the Americas and the resulting increased use of GLY correspond with amphibian population decline?

(vii) Are there as yet any 'signs' for negative impacts on amphibian populations in Germany as a result of an increased deployment of GLY in conventional agriculture?

Eventually, we summarised and discussed about

(viii) what kind of data is lacking with regard to obtaining a more conclusive picture of effects of GLY and its formulations on amphibians.

Results

(i) GLY and AMPA monitoring data are sparse, perhaps mainly because analysis is relatively expensive. Only data on aquatic habitats can be directly related to effects on amphibians because specific studies on, for instance, effects of contaminated soil are lacking. Maximum GLY concentrations, which have been found in the environment are 0.7 mg a.e./L (a.e. = acid equivalent) in small water bodies next to HR soybean cultivations in Argentina and 1.95 mg a.e./L in a forest pond after aerial applications in Canada at approved rates. Worst-case expected environmental concentration (EEC) for surface waters from national agencies are 1.44 mg a.e./L for Canada (where aerial applications are approved) and 0.9 mg a.e./L for Germany (drift rate into water during application without buffer strip). Some scientists calculated higher EEC up to 7.6 mg a.e./L for direct over-spraying of shallow water bodies. AMPA has been found at lower concentrations, but at higher frequencies. No EEC for AMPA is available. GLY and AMPA are regularly present in environmental samples of the Americas and Europe, but usually at low levels. Maximum GLY concentrations in the environment may be higher than those found because sampling usually did not take place directly after application or first heavy rainfalls after application. Maximum GLY concentrations are of main interest because toxic effects of GBH on tadpoles mainly occur within the first 24h. The published worst-case scenarios seem to represent good estimates and, apparently, can be used for risk assessment concerning amphibians. For Germany, the worst-case EEC of 0.9 mg a.e./L should be considered, but buffer strips of at least 5 m are required in most

German states, which should reduce the GLY concentration in the water to about 0.005 mg a.e./L. Nevertheless, detailed information on real contamination levels of aquatic and terrestrial amphibian habitats is widely lacking and small ephemeral water bodies like puddles or flooded fields and flooded pastures are not protected by buffer strips, but these are often used by several amphibian species for reproduction. Furthermore, the main problem remains that measured GLY concentrations are only proxies for GBH contamination, but added surfactants are mainly responsible for adverse effects.

(ii) Most studies used anuran larvae (tadpoles) as test organisms and little is known about effects on terrestrial life-stages as well as the other two amphibian orders (Caudata and Gymnophiona; i.e. salamanders/newts and caecilians). Amphibian toxicity studies on AMPA are lacking. Reported toxic effects on tadpoles include – besides increased mortality – damages of the gills and different malformations, inhibition of vital enzymes, release of oxidative stress and genotoxic effects. Chronic and delayed effects include delayed but also precipitated time to metamorphosis (which can lead to reduced fitness of metamorphs resulting in delayed mortality or increased mortality, when ephemeral ponds are drying out). Sometimes, effects were demonstrated at environmentally relevant concentrations. Most likely, added substances (surfactants) are responsible for adverse effects rather than GLY itself and species-specific responses have been observed. Hence, some GBH can be regarded as highly toxic for tadpoles at least of some anuran species while others are practically non-toxic. As already mentioned, adverse impacts of an increasing GBH use due to a possible introduction of HR crops on amphibian populations and communities can only be postulated for worst-case scenarios, mainly because of lacking data but also due to species, life-stage, formulation and application-specific (i.e. cultivation-specific) effects. The effects of long-term and regularly applications of GBH on wild amphibians should be monitored and evaluated site-specifically.

(iii) Several authors found different interactions of GLY and GBH with other stressors. In most cases, either another stressor enhanced the toxicity of the herbicide or the formulation enhanced the effect of the other stressor. Amphibian populations in anthropogenically influenced landscapes are usually affected by different stressors and, therefore, herbicide applications should not be regarded separately.

(iv) Potential exposure pathways are numerous and mainly include direct over-spraying of migrating and resting terrestrial life-stages, contact with contaminated plant material and soil, contamination of breeding ponds and ingestion of contaminated food or sediment.

(v) Conclusions concerning this question have to be hypothetical because of the limited data available for the Americas. To date, it remains unclear in which way different application timing in farming affect amphibians. In general, negative effects can only be postulated. Nevertheless, the commercial system of HR crop with its complementary non-selective herbicide (often GBH) allures to skip crop rotations. Hence, weed resistances with resulting equal or even higher herbicide application are likely including the re-use of selective herbicides. Few studies directly compared adverse effects of selective herbicides, which were replaced by the HR system and GBH on tadpoles, and arrived at the conclusion that some GBH are at least ranked among the most harmful herbicides for anuran larvae. In the future, such comparisons have to be conducted more detailed. However, if GBH are more dangerous to amphibians than the selective herbicides, which were replaced, remains unknown. To answer this question an intensive meta-analysis with more field data including extra laboratory, mesocosm and field studies and modelling have to be conducted. In general, GM crops facilitate the expansion of agriculture in less profitable land (further habitat destruction) and the aggregation of fields (further isolation and habitat destruction).

(vi) A causative correlation between increasing use of GLY and declining amphibian populations in the Americas cannot be stated because basic data are lacking (especially for South America) and, as already mentioned, especially because multiple factors – including their interactions – affect amphibian populations. Therefore, it is very difficult to assess the impact of a single stressor. Findings of the macroecological approach are furthermore limited due to low model fit.

(vii) For Germany, the results suppose that land use is more relevant for amphibian populations than pesticide applications including GLY applications. Hence, GBH apparently do not affect the considered populations, but also for Germany only limited data were available and no-tillage farming – where GBH are mainly used today – is still relatively rare in Germany (e.g. compared to the Americas). The impacts of no-tillage farming plus non-selective herbicide application on amphibians have to be compared with traditional farming methods. At a rough estimate, both farming methods have a similar potential to harm individuals, but it remains unclear whether they can affect entire amphibian populations, especially nationwide.

(viii) Missing data include some basic studies on possible effects of GLY and GBH on amphibians, the mentioned comparison of the impacts of non-selective and selective herbicides on amphibians, detailed data on the presence of amphibians in agricultural areas and their habitats within and information on real-world concentrations of GLY, AMPA and especially the added substances in aquatic but also terrestrial amphibian habitats. An analysis of deficits has been conducted.

Conclusions

Most agrochemicals – if fertilisers or pesticides – can affect amphibian individuals, which live within agricultural areas, but not necessarily with consequences at the population level. Population viability analysis based on sufficient data and/or long-term field monitoring is necessary to investigate potential impacts on amphibian populations. Some GBH, in particular those formulations with added tallowamine surfactants, apparently ranked among the most harmful pesticides for amphibians – in so far as this is known. Nevertheless, risks for amphibian populations due to GLY use cannot be named *per se* because species, life-stage, formulation and application-specific reactions have been observed. Hence, approval for GM crops with herbicide resistance in Germany should be accompanied by basic research and a monitoring that is able to identify effects on nearby amphibian populations. Likewise, the already conducted no-tillage farming has to be assessed with regard to amphibians. The EU and the German law already provide for provisions to monitor GM crops and prohibit any agrarian practices, which adversely affect amphibians.

II. ZUSAMMENFASSUNG

Hintergrund

Weltweit werden dramatische und unnatürliche Rückgänge von Amphibienpopulationen und selbst das Aussterben ganzer Arten beobachtet. Wissenschaftler versuchen seit langem herauszufinden, was die Gründe hierfür sind. Wahrscheinlich interagieren verschiedene Faktoren. Kontamination der Umwelt, z.B. durch Pestizidanwendungen, ist eine der diskutierten Ursachen. Daher wurde in diesem Gutachten darauf eingegangen, inwiefern der rezente Wandel von der konventionellen hin zur Landwirtschaft mit gentechnisch veränderten Pflanzen (GVP) Amphibien beeinträchtigen kann. Dieser Wechsel geht mit unterschiedlichen Herbizideinsätzen einher (hin zum exklusiven Gebrauch von Totalherbiziden), kann aber auch zu einer weiteren Zusammenlegung und Ausweitung von Agrarland führen. In Amerika werden in manchen Kulturen fast nur noch GVP angebaut. So beträgt der Anteil von GVP beispielsweise in Soja- und Baumwollkulturen bereits 81%. In Europa, speziell in Deutschland, werden momentan fast keine GVP angepflanzt. Dieses Gutachten behandelt hauptsächlich die potenziellen Auswirkungen, die unterschiedliche Herbizidanwendung beim GVP-Anbau im Vergleich zur konventionellen Landwirtschaft auf Amphibien haben kann. Der Großteil der heute angebauten GVP ist herbizidresistent, was bedeutet, dass diese Pflanzen nicht-selektive Herbizide (auch Breitband- oder Totalherbizide genannt) bei der Unkrautbekämpfung bis zu einem gewissen Grad tolerieren. Daher geht ein steigender Anbau von GVP auch mit einem steigenden Einsatz von Totalherbiziden in der Landwirtschaft einher. Meist ist Glyphosat (GLY) der aktive Wirkstoff in diesen Herbiziden. Durch den Einsatz von herbizidresistenten GVP sollen Applikationen selektiver Herbizide gänzlich ersetzt oder auf ein Minimum reduziert werden. Vor allem soll es im Vergleich zur konventionellen Landwirtschaft zu einer Reduzierung des kumulativen Gebrauchs von Herbiziden kommen. Die relative Umweltverträglichkeit von GLY im Vergleich zu anderen Herbiziden wird häufig hervorgehoben. Obwohl ein GVP-Anbau in den ersten Jahren tatsächlich die genannten Vorteile mit sich bringt, zeigt die praktische Erfahrung aus Amerika (wo GVP bereits seit fast zwei Jahrzehnten im großen Stil angebaut werden), dass es dadurch meist zu einer Vernachlässigung der Fruchtfolge kommt. Da die meist GLY-haltigen Totalherbizide folglich über lange Zeit auf denselben Flächen appliziert werden, haben sich GLY-resistente Unkräuter entwickelt, welche durch einen gesteigerten Totalherbizideinsatz oder aber durch einen zusätzlichen Einsatz selektiver Herbizide bekämpft werden müssen. Es scheint, dass eine Abnahme des kumulativen Herbizideinsatzes in der Praxis nicht langfristig erreicht werden kann (obwohl Resistenzen bei Unkräutern kein exklusives Problem von Totalherbiziden, sondern zumeist das Resultat einer nicht nachhaltigen Landwirtschaft sind). Im Hinblick auf die genannte Umweltverträglichkeit von GLY ergibt sich zudem das Problem, dass den im Feld angewandten Formulierungen in fast allen Fällen Netzmittel und andere Stoffe beigemengt sind, welche meist toxischer sind als der aktive Wirkstoff selbst.

Wir führten daher eine breite Literatur- und Datenbankrecherche durch, um folgende Schlüsselfragen zu beantworten:

(i) Welche Konzentrationen von GLY und von seinem Hauptmetaboliten Aminomethylphosphonsäure (AMPA) lassen sich in der Umwelt finden?

(ii) Was wissen wir aktuell über die Effekte von GLY und seinen Formulierungen auf Amphibien?

(iii) Gibt es Wechselwirkungen mit abiotischen und biotischen Stressoren?

(iv) Welche Expositionspfade bestehen für verschiedene Lebensstadien der Amphibien?

(v) Welche Auswirkungen hat ein exklusiver Einsatz von Totalherbiziden beim GVP-Anbau im Vergleich zu dem Einsatz selektiver Herbizide in der konventionellen Landwirtschaft?

Zudem wurde eine statistische, makroökologische Auswertung vorgenommen, um folgende Fragen näher zu beleuchten:

(vi) Gibt es Zusammenhänge zwischen dem Landwirtschaftswandel in Amerika, welcher mit einer steigenden Anwendung glyphosatbasierter Herbizide (GBH) einherging und geht, und beobachteten Rückgängen von Amphibienpopulationen?

(vii) Gibt es heute bereits in Deutschland Anzeichen dafür, dass ein auch hier steigender Einsatz von GBH in der konventionellen Landwirtschaft einen negativen Einfluss auf Amphibienpopulationen ausübt?

Abschließend wurde zusammengetragen und diskutiert,

(viii) welche Datengrundlagen fehlen, um ein schlüssiges Bild von den Wirkungen von GLY und seinen Formulierungen auf Amphibien zu erhalten

Ergebnisse

(i) Die Datenlage zu Umweltkonzentrationen von GLY und dem Hauptmetaboliten AMPA ist spärlich, wahrscheinlich hauptsächlich deshalb, weil die benötigten Laboranalysen verhältnismäßig teuer sind. Nur GLY- und AMPA-Konzentrationen in Oberflächengewässern können mit potenziellen Auswirkungen auf Amphibien in Beziehung gesetzt werden, da spezielle Studien über Auswirkungen von etwa kontaminiertem Boden fehlen. Maximale Umweltkonzentrationen von GLY sind 0,7 mg a.e./l (a.e. = „acid equivalent“ = Säureäquivalent) in einem benachbarten Gewässer zu GV-Sojafeldern in Argentinien und 1,95 mg a.e./l in einem Waldtümpel in Kanada nach (legaler) Ausbringung eines GBH mit dem Flugzeug. Vorausgesagte Umweltkonzentrationen (Expected Environmental Concentrations = EEC) in Oberflächengewässern sind 1,44 mg a.e./l für Kanada (wo Ausbringung per Flugzeug erlaubt ist) und 0,9 mg a.e./l für Deutschland (Drift bei Anwendung ohne Einhaltung eines Gewässerrandstreifens). Manche Wissenschaftler kalkulierten höhere Werte, bis zu 7,6 mg a.e./l nach direkter Applikation über einem sehr flachen Gewässer. AMPA wurde zumeist in niedrigeren Konzentrationen als GLY, jedoch häufiger nachgewiesen. In der Literatur fanden sich keine EEC für AMPA. GLY und AMPA sind in Umweltproben aus Amerika und Europa regelmäßig vertreten, jedoch zumeist in relativ niedrigen Konzentrationen. Jedoch können die tatsächlichen maximalen Konzentrationen in der Umwelt höher liegen, da die Gewässer zumeist nicht direkt nach einer Herbizidapplikation oder nach den ersten schweren Regenfällen nach der Applikation beprobt wurden. Maximale Konzentrationen von GLY und AMPA sind deswegen von Interesse, weil akuttoxische Effekte bei Kaulquappen meistens innerhalb der ersten 24 Stunden beobachtet wurden. Die vorausgesagten Umweltkonzentrationen für GLY repräsentieren gute Abschätzungen für die maximale Konzentration des Stoffes in der Umwelt und können für eine Risikoanalyse herangezogen werden. Für Deutschland sollten die kalkulierten 0,9 mg a.e./l als „worst-case-scenario" angenommen werden, jedoch müssen in den meisten Bundesländern Gewässerrandstreifen von mindestens 5 m eingehalten werden, welche die GLY-Konzentration im Wasser auf geschätzt etwa 0,005 mg a.e./l verringern sollten. Nichtsdestotrotz fehlen Detailinformationen zu der realen Kontamination der aquatischen und terrestrischen Lebensräume von Amphibien und des Weiteren werden sehr kleine ephemere Gewässer wie Pfützen oder aber überflutete Felder und Wiesen nicht durch Gewässerrandstreifen geschützt. Diese werden jedoch von vielen Amphibienarten häufig zur Reproduktion genutzt. Zudem besteht das Hauptproblem, dass GLY-Konzentrationen nur eine Abschätzung für eine Kontamination mit der jeweils applizierten Formulierung darstellen, jedoch beigemengte Netzmittel hauptsächlich für schädliche Wirkungen verantwortlich sind.

(ii) Testorganismen in den meisten Studien waren Kaulquappen und es ist rezent sehr wenig über Effekte auf terrestrische Lebensstadien von Anuren und im Allgemeinen auf die beiden anderen Amphibien-Ordnungen bekannt (Caudata und Gymnophiona; d.h. Schwanzlurche und Blindwühlen). Spezielle Toxizitätsstudien mit AMPA fehlen gänzlich. An toxischen Effekten bei Kaulquappen wurden – neben erhöhter Mortalität – Schäden an den Kiemen und verschiedene Missbildungen, Hemmung von Enzymen, oxidativer Stress und mutagene Effekte beobachtet. Beobachtete chronische und verzögerte Effekte waren verkürzte oder verlängerte Dauer bis zur Metamorphose (was zu reduzierter Fitness von Metamorphlingen und eine dadurch verzögerte erhöhte Mortalität einerseits und eine erhöhte Mortalität durch das Austrocknen ephemerer Gewässer andererseits führen kann). In manchen Fällen traten Effekte bei umweltrelevanten Konzentrationen auf. Verantwortlich für schädliche Wirkungen sind höchstwahrscheinlich die beigemengten Stoffe (Netzmittel) und nicht der aktive Wirkstoff selbst. Zudem waren die Reaktionen meist artspezifisch. Manche GBH können als hochtoxisch für Kaulquappen zumindest mancher Arten angesehen werden, andere GBH als praktisch nicht toxisch. Davon kann nicht abgeleitet werden, dass ein Anbau von GVP mit Herbizidresistenz *per se* schädliche Effekte auf Amphibien hat, da grundlegende Felddaten fehlen und auch, weil Effekte oftmals nicht nur artspezifisch sind, sondern auch vom jeweiligen Amphibienlebensstadium, der Formulierung und der Applikationsmethode abhängen. Die Effekte eines langfristigen GVP-Anbaus mit fast exklusivem Gebrauch von GBH auf Amphibienpopulationen sollten daher lokal beobachtet und bewertet werden.

(iii) Viele Autoren fanden Interaktionen zwischen GLY und GBH mit anderen Stressoren. In den meisten Fällen verstärkte ein zusätzlicher Stressor die Toxizität des Herbizids oder das Herbizid verstärkte den Effekt des zusätzlichen Stressors. Da Amphibienpopulationen in der Kulturlandschaft im Normalfall einer Vielzahl biotischer und abiotischer Stressoren ausgesetzt sind, sollte der Einfluss von Herbiziden auch nicht alleinstehend betrachtet werden.

(iv) Potenzielle Expositionspfade sind vielfältig und beinhalten hauptsächlich direktes Übersprühen wandernder oder rastender Tiere und solcher, die ein Feld als Teilzeitlebensraum akzeptieren, Kontakt mit kontaminiertem Pflanzenmaterial und Boden, die Kontamination von Laichgewässern und die Aufnahme über kontaminierte Nahrung als auch Sediment.

(v) Aussagen zu dieser Fragestellung müssen hypothetischer Natur bleiben, da für Amerika nur sehr begrenzte Datensätze zur Verfügung standen. Daher bleibt es derzeit letztendlich unklar, inwieweit unterschiedliche Applikationszeitpunkte Auswirkungen auf Amphibien besitzen und negative Effekte können insgesamt nur postuliert werden. Nichtsdestotrotz beinhaltet das kommerzielle System, welches aus Saatgut von GVP mit komplementärem Totalherbizid besteht, die Gefahr, dass durch nicht nachhaltige Landwirtschaft Resistenzen bei Unkräutern entstehen und dadurch gleiche und eventuell sogar höhere Herbizidmengen – inklusive selektiver Herbizide – eingesetzt werden müssen. Wenige Studien verglichen den Einfluss selektiver und nicht-selektiver Herbizide auf Anurenlarven direkt. Die Autoren schlussfolgerten, dass manche GBH zumindest zu den toxischsten Herbiziden für Kaulquappen zählen. Zukünftig sollten detaillierte Untersuchungen zu diesem Thema stattfinden. Ob GBH insgesamt gefährlicher für Amphibien sind, bleibt zurzeit dennoch ungewiss. Um diese Frage zu beantworten, müsste eine ausgedehnte Metaanalyse mit einem großen Felddatensatz und speziell durchgeführten Labor-, Mesokosmos-, Feldstudien und Modellierungen durchgeführt werden. Allgemein erleichtert der GVP-Anbau die Vergrößerung und Zusammenlegung von Feldern, was zu weiterer Habitatzerstörung und Isolierung von Amphibienpopulationen führen kann.

(vi) Ein kausaler Zusammenhang zwischen dem steigenden Einsatz von GLY und Bestandsrückgängen bei Amphibien in Amerika kann nicht hergestellt werden, weil Basisdaten fehlen (v.a. für Südamerika) und hauptsächlich weil verschiedenste Faktoren Amphibienpopulationen beeinflussen. Daher ist der Einfluss eines einzelnen Stressors sehr schwierig zu betrachten. Die Aussagekraft der Resultate unserer statistischen Auswertungen ist zudem durch schlechte Modellgüte limitiert.

(vii) In Deutschland scheint die umgebende Landnutzung für Amphibienpopulationen relevanter zu sein als Pestizidapplikationen (inklusive GLY-Anwendungen). Scheinbar haben GBH keine messbaren Effekte auf die betrachteten Populationen, jedoch waren auch für Deutschland nur limitierte Daten erhältlich und die pfluglose Feldbearbeitung – bei der GBH rezent hauptsächlich zur Anwendung kommen – ist im Vergleich zu etwa Amerika seltener. Potenzielle Effekte des Einsatzes von Totalherbiziden bei der pfluglosen Bodenbearbeitung sollten mit denen der traditionellen mechanischen Bodenbearbeitung verglichen werden. Nach einer ersten Einschätzung sollten jedoch beide Methoden vergleichbare negative Wirkungen mit sich bringen.

(viii) Grundlagenforschung zu den Effekten von GLY und seinen Formulierungen auf Amphibien sind weiterhin erforderlich. Zudem sollte versucht werden, Einflüsse von selektiven und nicht-selektiven Herbiziden gegenüberzustellen. Des Weiteren fehlen Daten zum Vorkommen und zu den Habitaten von Amphibienpopulationen in landwirtschaftlich genutzten Gegenden, zu Umweltkonzentrationen von GLY und AMPA und speziell zu Umweltkonzentration von den Formulierungen beigemengten Substanzen in aquatischen als auch terrestrischen Lebensräumen. Daher wurde im Rahmen dieses Gutachtens eine Defizitanalyse durchgeführt.

Fazit

Die meisten Agrochemikalien, ob Düngemittel oder Pestizide, besitzen das Potenzial, Amphibien, welche in der Kulturlandschaft leben, zu schädigen. Jedoch hat dies nicht zwangsweise Auswirkungen auf die Population. Populationsmodelle – basierend auf einer guten Datenbasis – und/oder Langzeitmonitoring im Feld sind notwendig, um Effekte auf der Populationsebene zu untersuchen. Manche GBH, insbesondere solche mit tallowaminhaltigen Netzmitteln, gehören scheinbar zu den toxischsten Pestiziden für Amphibien, die man kennt. Jedoch können Risiken einer GLY-Nutzung für Amphibienpopulationen nicht *per se* genannt werden, da es art-, lebensstadien-, formulierungs- und applikationsspezifische Reaktionen gibt. Zulassungen zum Anbau von GVP mit Herbizidresistenz in Deutschland sollten daher mit weiterer Grundlagenforschung und einem Monitoring verbunden werden, das mögliche Auswirkungen des Totalherbizids auf benachbarte Amphibienpopulationen erfassen kann. Dies gilt generell auch für den Anbau konventioneller Kulturen, wenn Totalherbizide bei der pfluglosen Feldbearbeitung eingesetzt werden. Zudem sollte der Totalherbizideinsatz bei der pfluglosen Feldbearbeitung bezüglich seiner Auswirkungen auf Amphibien untersucht werden. Das EU-Recht wie auch das deutsche Recht enthalten Vorgaben für das Monitoring von GVP und um jedwede landwirtschaftliche Praxis, die zu schädlichen Auswirkungen auf Amphibien führt, verbieten zu können.

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V. ABBREVIATIONS USED

µg	microgram (10^{-6} g)
a.e.	acid equivalent
a.i.	active ingredient
AMPA	aminomethylphosphonic acid
BfN	Bundesamt für Naturschutz (Federal Agency for Nature Conservation, Germany)
BNatSchG	Bundesnaturschutzgesetz (federal law on nature protection)
Bt	*Bacillus thuringiensis*
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety, Germany)
cf.	compare (Latin: confer)
cm	centimetre
CO_2	chemical formula for carbon dioxide
DFG	Deutsche Forschungsgemeinschaft (German Research Foundation)
e.g.	for example (Latin: exempli gratia)
et al.	and others (Latin: et alii/et aliae)
EC	European Commission
EC50	half maximal Effective Concentration induces a response halfway between the baseline and maximum after some specified exposure time
EEC	Expected Environmental Concentration
EU	European Union
FAO	Food and Agriculture Organisation of the United Nations
Fig.	Figure
g	gram
GBH	glyphosate-based herbicide(s)
GLY	glyphosate
GM	genetically modified
GMO	genetically modified organism
GT	glyphosate tolerant
h	hours
ha	hectare
HR	herbicide resistant
i.e.	that is (Latin: id est)
ISAAA	International Service for the Acquisition of Biotech Applications
kg	kilogram (10^3 g)
L	litre

LC50	median lethal concentration required to kill half the members of tested organisms after specified test duration
m	metre
mg	milligram (10^{-3} g)
Mn	chemical code for manganese
MON	Monsanto
NABU	Naturschutzbund Deutschland (Nature and Biodiversity Conservation Union, Germany)
NGO	non-governmental organisation
NPE	nonylphenol polyethoxylate
OH^-	chemical formula of the hydroxide ion
p.	page
pp.	pages
ppb	parts-per-billion (10^{-9}, quantity-per-quantity measure with no associated units of measurement)
ppm	parts-per-million (10^{-6}, quantity-per-quantity measure with no associated units of measurement)
POEA	polyethoxylated tallowamine
SE	standard error
SRU	Sachverständigenrat für Umweltfragen (German Council of Environmental Advisors)
Subpara.	Subparagraph
USA	United States of America
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
UV	ultraviolet

VI. REPORT

1. Introduction

Glyphosate (GLY) is the active ingredient (a.i.) in many broad-spectrum herbicide formulations. It is understood to have mortal effects on most species of green plants by inhibiting the biosynthesis of aromatic amino acids (e.g. GIESY et al. 2000; MONSANTO 2005; DILL et al. 2010; see also chapter 3). Environmental risk assessment concerning GLY is highly relevant as it has become the dominant herbicide worldwide, mainly due to the success of genetically modified (GM) crops with herbicide resistance, so called HR crops (DUKE & POWLES 2008). The United States Environmental Protection Agency (USEPA), as well as the European Commission, considers GLY to be relatively harmless to the environment compared with other active ingredients. This opinion is based on the known environmental fate of GLY and ecotoxicological laboratory standard tests with it (USEPA 1993; EUROPEAN COMISSON 2000, 2002). However, amphibians do not belong to the standard test organisms. Mainly based on laboratory but also field studies, some researchers consider especially anuran amphibians (that is frogs and toads) to be non-target organisms affected by the wide use of GLY. Due to their life history and physiology, it may be expected that these animals are particularly sensitive to GLY and its formulations (e.g. SMITH 2001; RELYEA 2004; CAUBLE & WAGNER 2005; RELYEA 2005a,b,c; GASCON et al. 2007; RELYEA & JONES 2009; JONES et al. 2010; WILLIAMS & SEMLITSCH 2010; LAJMANOVICH et al. 2011). Nevertheless, the discussion of the potential impact of GLY on amphibian species and populations under environmental conditions remains controversial. Some studies (e.g. EDGINTON et al. 2004; THOMPSON et al. 2004; WOJTASZEK et al. 2004; BERNAL et al. 2009b), in conjunction with the reviews by GIESY et al. (2000), SOLOMON & THOMPSON (2003) and LANGELAND (2006), imply that deleterious effects on adult or larval amphibians and other aquatic taxa are not to be expected by the use of GLY under realistic environmental exposures and 'normal-use' scenarios. In contrast to the active ingredient GLY, several studies have demonstrated that commercial glyphosate-based herbicides (GBH) are more toxic due to the toxicity or interactions of added components, namely surfactants. The surfactant POEA (polyethoxylated tallowamine) could be a principal toxicant responsible for lethal and sublethal effects on amphibian larvae (e.g. MANN & BIDWELL 1999; PERKINS et al. 2000; LAJMANOVICH et al. 2003; CHEN et al. 2004; HOWE et al. 2004; EDGINTON et al. 2004; BERNAL et al. 2009a) and other aquatic taxa (e.g. FOLMAR et al. 1979; GIESY et al. 2000; PARTEARROYO et al. 2001; HALLER & STOCKER 2003; BRINGOLF et al. 2007). Due to delays, reevaluation of GLY will be conducted complying only with the old and not the new requirements of the European Union (namely the more strictly European Plant Protection Products Regulation 1107/2009 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:EN:PDF) and the

European Pesticides Framework Directive 2009/128/EC (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0071:0086:EN:PDF). Still, added components will be disregarded. ANTONIOU et al. (2011)[1] hold especially responsible the German Federal Office of Consumer Protection and Food Safety ('Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL) for playing down findings of serious risks of GLY and its formulations including endocrine disruption, carcinogenic effects, genotoxicity, neurotoxicity and especially teratogenic effects resulting in observed birth defects.

The public opinion of the introduction of genetically modified organisms (GMO) is highly controversial. Concerning HR crops, a group including profiting industrials, farmers, researchers, and decision makers refers to the economic and ecological benefits of the supposed decrease in absolute and per hectare herbicide use (e.g. BROOKES & BARFOOT 2009 for the USA; CERDEIRA & DUKE 2006; CERDEIRA et al. 2007 for South America). Its adversary unites conservationists (e.g. NGOs), but likewise farmers, researchers and politicians; they question these benefits because of suspected negative long-term impacts on the environment and the acquired GLY resistance of some weeds (reviewed by POWLES & WILCUT 2008) leading to the additional re-use of conventional herbicides in HR crop cultivation which could cause equal or increasing absolute and per hectare use of herbicides (see BENBROOK 2009, 2012). According to the German Council of Environmental Advisors ('Sachverständigenrat für Umweltfragen', SRU) (2008), it remains unclear if HR crop cultivation in the USA really reduced the use of herbicides. The German Research Foundation ('Deutsche Forschungsgemeinschaft', DFG) (2011: p. 73) stated that HR soybean cultivation in the USA slightly reduced herbicide consumption while in South America herbicide consumption clearly increased due to the adoption of HR crops. According to the DFG (2011), the reason for the increase in South America should be the simultaneously increase of no-tillage farming[2]. However, the DFG (2011) does not cite any specific reference here and altogether lists only a couple of references in its brochure. The brochure has been recently criticised by TAUBE et al. (2011) and a response of the authors has been provided too (BROER et al. 2011). Regarding herbicide resistant weeds[3], BROER et al. (2011) and the DFG (2011) rightly stated that the development of resistant weeds is not a specific problem with GM crops but the result of non-sustainable pesticide use. However, just the system of HR crops and their complementary broad-spectrum herbicides favours monocultures with only one crop and trait (i.e. only one applied a.i.). Hence, some authors question if the cumulative herbicide use really decrease over the long-term. Taking the USA as example, a reduced herbicide application could

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1 Note that ANTONIOU et al. (2011) is not a peer-reviewed report.

2 PHILLIPS et al. (1980) defined the no-tillage system as "one in which the crop is planted either entirely without tillage or with just sufficient tillage to allow placement and coverage of the seed with soil to allow it to germinate and emerge. Usually no further cultivation is done before harvesting. Weeds and other competing vegetation are controlled by chemical herbicides. Soil amendments, such as lime and fertilizer, are applied to the soil surface." Today, mainly non-selective herbicides like glyphosate formulations were applied for controlling of weeds in no-tillage farming (e.g. RAUBUCH & SCHIEFERSTEIN 2002). The discussion on the benefits and drawbacks of no-tillage farming is controversial (see also chapter 6).

3 'Herbicide resistant' and 'herbicide tolerant' are synonymous.

only be observed for the initial years of GM crop cultivation, but over the long-term application rates were equal (DALE et al. 2002) or even higher than in conventional cultivations (BENBROOK 2009, 2012). Conversely, other authors stated the contrary, i.e. a significantly decreased cumulative herbicide use (e.g. KEMPKEN & KEMPKEN 2004). However, meanwhile HR crops with (multiple) herbicide resistances other than to GLY are developed (e.g. WRIGHT et al. 2010; PETERSON et al. 2011). This could be seen as a confession that the reduction of herbicide usage, observed at the beginning of GLY-resistant crop cultivation, cannot be realised in the long-term in the real-world. If the problem of resistant weeds can be solved by adding new HR traits into new crops remains highly disputable (see EAGAN et al. 2011 vs. WRIGHT et al. 2011).

Altogether, some scientists blame each other not to be neutral in the discussion on benefits and potential drawbacks of HR crops. For example, BROER et al. (2011) stated that GLY has the potential to be beneficial to the environment due to favourable ecotoxicological traits (BROER et al. 2011: p. 5). However, to document this statement they only cited one article from a book that has arisen from the conference entitled "Biotechnology, Science and Modern Agriculture: a New Industry at the Dawn of the Century", convened by the International Consortium on Agricultural Biotechnology Research (ICABR). Among others, the recent conference of the ICABR is sponsored by different companies that produce both GMO and broad-spectrum herbicides (http://www.economia.uniroma2.it/icabr-conference/sarea.php?p=12&sa=165). Conversely, TAUBE et al. (2011) cited so called 'grey literature', i.e. not peer-reviewed. This shows that it is very difficult to get neutral information in this debate.

The Directive 2001/18/EC of the European Parliament and the Council requires a risk assessment of GMO including direct, indirect, immediate and delayed effects on the environment (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:106:0001:0038:EN:PDF). This risk assessment should also include cumulative long-term effects that are especially relevant for cultivations with a rotation of crops but not traits (e.g. HR maize follows HR canola). The Federal Agency for Nature Conservation, Germany ('Bundesamt für Naturschutz', BfN), is involved in the risk assessment and the admission procedure of GMO in Germany[4]. Hence, the BfN has commissioned an expert opinion from the Department of Biogeography, Trier University, on the possible correlation of the worldwide amphibian decline and the increasing use of GLY in the agrarian industry. Furthermore, the topic of this expert opinion is in accord with the decisions of the COUNCIL OF THE EUROPEAN UNION (2008) that declares "… the need to study the potential consequences for the environment of changes in the use of herbicides caused by herbicide-tolerant GMPs [genetically modified plants]".

The main focus of this expert opinion is the cultivation of HR crops, the application of the complementary broad-spectrum herbicide GLY and possible drawbacks for amphibians. It explicitly excludes the controversial discussion of possible health damages caused by GM food as well as of

[4] See § 16 (4) of the German Genetic Engineering Act. http://www.gesetze-im-internet.de/bundesrecht/gentg/gesamt.pdf

ethics related to genetic engineering. This paper only peripherally discusses the claimed benefits and potential risks of GMO including HR crops in general. For the purposes of comparison, the following regions are especially considered: (i) North and South America, where GM crop cultivation has strongly increased over the last two decades and (ii) Germany, where the proportion of GM plants in agriculture is as yet negligible[5].

Key questions of this expert opinion are as follows:

- Which concentrations of GLY and its main metabolite can be found in the environment?
- What do we actually know about the impacts of GLY and its formulations on amphibians?
- Can there be identified effects on amphibians as a result of the use of GLY coupled with biotic or abiotic stressors?
- Are amphibians differently affected by the cultivation of conventional crops compared with the HR crops especially with respect to the different weed management systems?
- What are possible exposure pathways to different amphibian life-stages?
- Does the agricultural change in the Americas and the resulting increased use of GLY correspond with amphibian population decline?
- Are there as yet any 'signs' for negative impacts on amphibian populations in Germany as a result of an increased deployment of GLY in the conventional agriculture?
- What kind of data is missing with regard to obtaining a more conclusive picture of effects of GLY to amphibians?

2. Amphibian decline and extinction

It is no longer deniable that amphibian populations are declining at the global scale (MENDELSON et al. 2006; GASCON et al. 2007; STUART et al. 2008). First discussed in 1989 at the ‘First World Congress of Herpetology’ (BARINAGA 1990), research activities have since focused on global patterns describing and understanding this problem (e.g. ALFORD & RICHARDS 1999; BLAUSTEIN & KIESECKER 2002; COLLINS & STORFER 2003). However, many drastic declines had occurred before so that in general our understanding remains poor (e.g. HOULAHAN et al. 2000; LA MARCA et al. 2005; STUART et al. 2008). Complex interactions of different factors are apparently at work. COLLINS & STORFER (2003) sorted six leading hypotheses into two classes. Class I hypotheses (alien species, over-exploitation, land use change) include causes that have negatively affected amphibians for more than a century, whereas their class II hypotheses (global change including increased UV radiation and climate change, contaminants, emerging infectious diseases) are considered more recent, with their greatest influence dating from the middle of the

[5] For an index of fields with admitted cultivation of GM plants in Germany see

last century. According to these authors, factors allocable to the class II hypothesis include complex and subtle mechanistic underpinnings and interactions of different ecological and evolutionary variables are possible (they consequently may be grouped with class I hypotheses).

Some biologists call amphibians, especially frogs and toads (Anura) 'excellent bioindicators' (e.g. BLAUSTEIN 1994; BLAUSTEIN & WAKE 1995) for changes at the local scale, because the terrestrial habitats of adults of many species are not far away from the aquatic larval developmental sites (BLAUSTEIN et al. 1994a). BLAUSTEIN & JOHNSON (2003) stated that their permeable, thin skin and shell-less eggs are directly exposed to the environment and readily absorb substances. Further, they pointed out that the biphasic life cycle of many amphibian species with aquatic larval and terrestrial adult stages make them vulnerable to both aquatic and terrestrial environmental change. Moreover, due to their biology and physiology, amphibians are regarded as especially sensitive to atmospheric change (e.g. precipitation, UV radiation; BLAUSTEIN & JOHNSON 2003). Together with their rudimentary immune system (WAKE & VREDENBURG 2008) and the circumstance that most anuran amphibians (versus newts, salamanders etc.) are phytophagous as larvae and carnivorous as adults, they should be especially sensitive to environmental impact beyond the above mentioned including anthropogenic environmental pollution (MORELL 1999).

In awareness of the global amphibian decline and the apparent high sensitivity of this group to environmental change and human impact, amphibians have earned their striking appellation as ‘canaries in a coal mine’ where the coal mine is equivalent to our planet. First used in an article called ‘Frogs as Canaries’ of the New York Times in 1990, numerous authors have continued using this metaphor in scientific literature and have hypothesised that amphibian declines may be the prelude to an environmental catastrophe that could affect many species and eventually ecosystems (e.g. COWEN 1990; VITT et al. 1990; MORELL 1999; HALLIDAY 2000; NORRIS 2007). Recently, WAKE & VREDENBURG (2006) asked "Are we in the midst of the sixth mass extinction?" in addition to five such events in prehistorical time which were naturally driven, while the expected on-going biodiversity crisis may be human-caused. More recently, KERBY et al. (2010) questioned the concern that widespread amphibian declines are early 'prophets' of broader environmental degradation that eventually will not only affect other species but also mankind. These authors applied the USEPA database ‘AQUIRE’ (Aquatic Toxicity Information Retrieval), representing thousands of toxicity tests, to compare the responses of amphibians to that of other taxonomic groups. They focused on chemical contaminants due to the possible role of chemical pollution in amphibian declines (e.g. BLAUSTEIN et al. 2003; COLLINS & STORFER 2003; COLLINS & CRUMP 2009). In their study, KERBY et al. (2010) estimated the ‘hazardous concentrations’ (HC50), i.e. the estimates in which 50% of species within a taxon exhibit at least 50% mortality (POSTHUMA et al. 2002). Amphibians were only the second most sensitive taxa to phenols and displayed relatively low to moderate sensitivity to pesticides, heavy metals and inorganics. The results show that amphibians are not particularly sensitive and may perhaps be

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http://apps2.bvl.bund.de/stareg_web/showflaechen.do better called the 'miners in a coal mine' (KERBY et al. 2010). As a consequence, population declines and extinctions may already have occurred in taxa much more sensitive than amphibians. Nevertheless, surveys on natural populations have shown correlations between amphibian population declines and the proximity to agricultural areas (e.g. HOULAHAN & FINDLAY 2003; DAVIDSON 2004). Supposed unnatural malformation rates have been reported from areas with intensive pesticide and fertiliser use (e.g. OUELLET et al. 1997; TAYLOR et al. 2005). Migration to ponds and spawning of most temperate amphibians occurs in spring and larval development in summer and therefore coincides with the application of pesticides and fertilisers on agricultural lands (MANN et al. 2009). They stated: "When considering these factors in addition to the large quantities of various herbicides, insecticides and fungicides presently used in agricultural production the resulting impacts on anurans (and perhaps other amphibians) have the potential to be significant."

No matter if amphibians are among the most reliable surrogates or not, researchers, conservationists etc. agree that these vertebrates are currently undergoing dramatic declines at the global scale, with the number of species threatened with extinction exceeding 30% of the almost 7,000 species known (STUART et al. 2008). HOULAHAN et al. (2000), BOSCH et al. (2007) and others have demonstrated that significant declines of both amphibian populations and species have occurred in Western Europe and there is a high need for immediate action to stop and reverse these trends. First, there are ethical issues concerning species losses. As in the case of the amphibians, we are apparently facing the high risk of the extinction of an entire vertebrate class (MENDELSON et al. 2006; GASCON et al. 2007). Second, the law obliges us to act. For example, many amphibian species are listed in the annexes of the European Habitats Directive and, for instance, in Germany, all amphibian species are at least listed as 'specially protected' ('besonders geschützt') under the Federal Ordinance on the Conservation of species ('Bundesartenschutzverordnung').[6] Apart from this, the anthropocentric viewpoint argues for immediate conservation action. In multiple ecosystems, amphibians belong to the top carnivores that are major consumers of invertebrates, especially arthropods and it has been advocated that amphibian declines have widespread consequences for food webs (WAKE 1991; COLÓN-GAUD et al. 2010 and references therein). Moreover, we know much about our own embryonic development based on meticulous studies on the amphibian ontogenesis (NORRIS 2007). Another cause may be that amphibians develop a variety of skin secretions which, although still poorly understood, potentially represent resources for the development of unique pharmaceutical products (e.g. DALY et al. 2000; NORRIS 2007). Most recently, anuran skin peptides have been identified as possessing a strong inhibition of the Human Immunodeficiency Virus Type 1 (HIV-1) (e.g. ROLLINS-SMITH 2009; WANG et al. 2010).

[6] http://www.bfn.de/0305_rechtsgrundlagen+M52087573ab0.html

3. Glyphosate

GLY is a phosphonomethyl derivative of the amino acid glycine and the active ingredient of many systemic non-selective herbicide formulations. Its official name under the International Union of Pure and Applied Chemistry (IUPAC) is 'N-(phosphonomethyl)glycine' (ROBERTS 1998). GLY is an odourless white crystalline solid. It is composed of one basic amino function plus three ionisable acid sites. Normally, GLY is used in the salt and not the acid form (DILL et al. 2010). Data on amounts consider the acid equivalents (a.e.) or the amount of active ingredient (a.i.).

3.1 Glyphosate's mode of action

GLY inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyses a key step in the synthesis of aromatic amino acids (e.g. AMRHEIN et al. 1980; ASPELIN 2003). A detailed description of GLY's mode of action has been provided by DILL (2005). Actually it is not entirely clear how the inhibition of the shikimate pathway kills the plants and whether inhibition of the EPSPS is the only herbicidal function of GLY (DUKE & POWLES 2008). To date, no other mode of action has been observed even when GLY was applied in high doses (NANDULA et al. 2007). However, HUBER (2009) and JOHAL & HUBER (2009) suppose that GLY additionally acts as chelator, i.e. GLY not only inhibits EPSPS by replacement of the enzyme substrate, but also by ligating the cofactor manganese.

3.2 Glyphosate use

First synthesized in 1950 by H. MARTIN at Cilag AG (Schaffhausen, Switzerland), GLY was tested for herbicidal activity in 1970 by J.E. FRANZ of Monsanto Company (St. Louis, USA) (FRANZ et al. 1997; DILL et al. 2010) and marketed in the USA only four years later (MONSANTO 2005). Use of GLY increased slowly during the following twenty years, but accelerated with the introduction of GM crops in 1996 (Fig. 1). Already in 2000, GLY was besides atrazine among the most widely used herbicides for weed control in the entire world (WOODBURN 2000) and today it is the world's leading herbicide (DUKE & POWLES 2008; see also chapter 3.7).

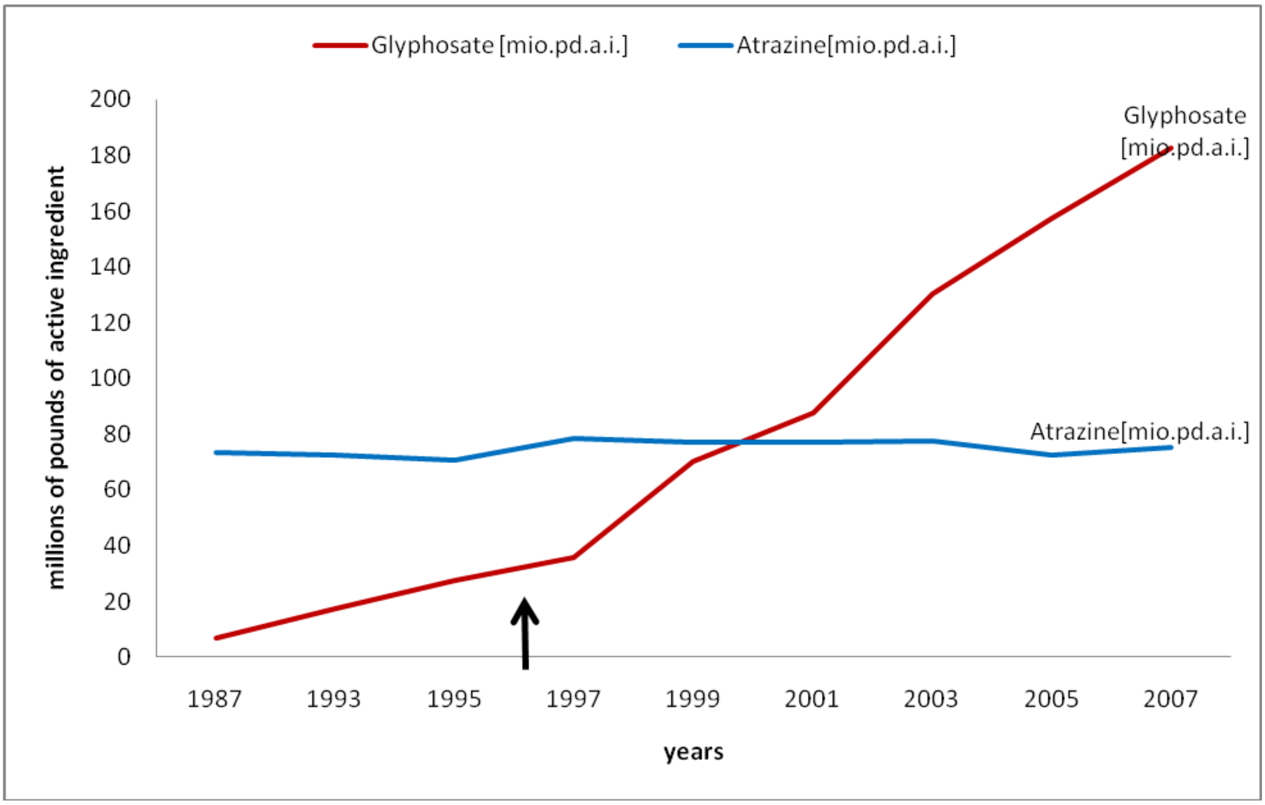

Fig. 1: Estimated GLY and atrazine application in the agricultural sector in the USA between 1987 and 2007
(based on ASPELIN 1997; ASPELIN & GRUBE 1999; DONALDSON et al. 2002; KIELY et al. 2004; GRUBE et al. 2011).
black arrow = GM crops were introduced in the USA in 1996; mio.pd.a.i. = millions of pounds of active ingredient.

The United States Department of Agriculture (USDA) stated that "U.S. farmers have adopted genetically engineered crops widely since their introduction in 1996, notwithstanding uncertainty about consumer acceptance and economic and environmental impacts" (USDA 2010), and the adoption of GM crops has rapidly grown and continues to do so (Fig. 2). On the global scale, GM crops were cultivated on about 124,000,000 hectares in 2008 (DFG 2010) and 134,000,000 hectares in 2009 (ISAAA 2010).

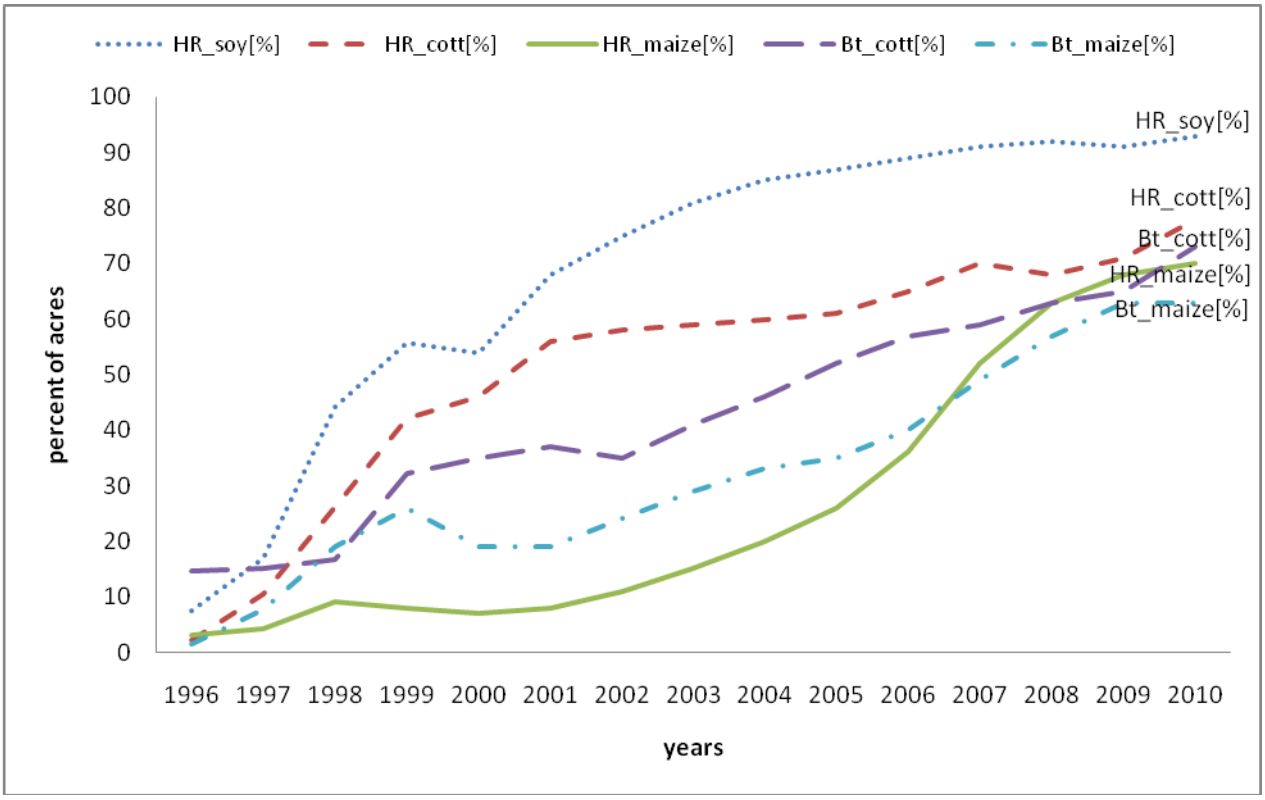

Fig. 2: Adoption of GM crops in the USA since 1996
(according to the USDA 2010).
HR_soy[%] = proportion of soya acres with HR soya; HR_cott[%] = proportion of cotton acres with HR cotton; HR_maize[%] = proportion of maize acres with HR maize; Bt_cott[%] = proportion of cotton acres with insect resistant cotton; Bt_maize[%] = proportion of maize acres with insect resistant maize.

Detailed information on the use of GLY in South America is lacking but it has undoubtedly increased along with the significant agricultural transformation driven by the adoption of GM crops (e.g. PENGUE 2004). Today nearly all of the soybean cultivated in Argentina and around 70% of that cultivated in Brazil is herbicide resistant with the vast majority being resistant to GLY (data refer to HR crops with single transformation events as well as stacked events; see chapter 3.7; ISAAA 2010). Detailed information on GLY consumption is also lacking for Germany where the proportion of GM crops is all but negligible. This applies especially to the years before 2004. At least in conventional agriculture the use of GLY had increased in Germany from 1,093 tons in 1993 to 2,745 tons in 2000 which is probably related to the increase of no-tillage farming in Germany (RAUBUCH & SCHIEFERSTEIN 2002). Since 2004, GLY has been among the pesticides with the highest sales in Germany (see chapter 3.6).

Interim conclusion

GLY use has exploded in the Americas since the introduction of HR crops. However, also in countries like Germany where the proportion of GM plants in agriculture is as yet negligible, GLY use has increased, probably simultaneously with the increasing no-tillage farming.

3.3 Introduction about effects on non-target organisms

Since EPSPS is not only present in plants, but in fungi and bacteria too (KISHORE & SHA 1988), the effect of GLY on the soil and on soil organisms was studied, but delivered different conclusions. For example, GLY neither deleteriously affects microbial biomass (i.e. numbers of bacteria, fungi and actinomycetes) nor soil respiration (i.e. the activity of the microorganisms) when applied to a conifer forest (STRATTON & STEWART 1992). GLY also did not affect nitrogen-cycling in two agricultural soils (MÜLLER et al. 1981). However, JAWORSKI (1972) already observed an inhibition of rhizobia at low GLY concentrations (1.7 ppm). In contrast to STRATTON & STEWART (1992) and MÜLLER et al. (1981), ROSLYCKY (1982) applied a long-term study design and found negative effects on microorganisms when GLY concentration in soil exceeds 1 µg/L. In general, soil microorganisms show different sensitivity towards GLY. Some are tolerant up to a certain level, degrade or even utilise GLY (see chapter 3.4.1). Due to microbial community shifts in soils with repeated GLY applications, more tolerant microorganisms remain that can use GLY as an available P source (ARAÚJO et al. 2003; SING & WALKER 2010; see chapter 3.4.1). However, beneficial fungi like mycorrhiza or entomopathogenic fungi (= that act as a parasite of harmful insects) belong to the less tolerant microorganisms (KREMER & MEANS 2009; MERTENS 2011). This highlights the importance of evaluating potential effects of HR crops on soil fertility. Taken together, the exposure of the soil microbial community to GLY appears to cause complex and varied responses (see chapter 7 for detailed information).

Based on official laboratory tests on both terrestrial and aquatic standard test organisms and according to the official categories defined by the USEPA (http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm), GLY isopropylamine salt is evaluated as ‘practically non-toxic’ and GLY acid as ‘practically non-toxic’ to ‘slightly toxic’ (DILL et al. 2010). In scientific studies, for instance, McCOMB et al. (2008) intraperitoneal injected GLY isopropylamine salt to nine species of wild terrestrial vertebrates. The authors considered a large margin of safety between dosages encountered after aerial applications of GLY in forests and dosages causing deleterious effects in the laboratory. However, such studies only evaluated the active ingredient GLY, but not the formulations used in the field. Usually, substances are added to GBH. For example, the popular formulation Roundup® is ‘moderately toxic’ to aquatic test organisms. It is about 40 to 50 times more toxic than the active ingredient (see Table 1, and for more information chapter 3.6).

Tab. 1: Comparison of the acute toxicity of GLY acid, GLY isopropylamine salt and Roundup Original® (MON 2139) including POEA as surfactant to some standard test organisms (modified after DILL et al. 2010).

Species	Exposure duration	GLY acid	GLY isopropylamine salt	GBH Roundup® (MON 2139)
Aquatic organisms		**LC50/EC 50 (mg a.e./L)**		
Rainbow Trout (*Oncorhynchus mykiss*)	96h	71.4 = ST	> 460 = PNT	1.3 = MT
Bluegill (*Lepomis macrochirus*)	96h	99.6 = ST	> 460 = PNT	2.4 = MT
Daphnia magna	48h	128 = PNT	428 = PNT	3.0 = MT
Terrestrial organisms		**LD 50 (units as indicated)**		
Rat (*Rattus norvegicus forma domestica*)	Single dose	> 4,275 mg a.e./kg body weight = PNT	-	1,550 mg a.e./kg body weight = ST
Bobwhite (*Colinus virginianus*)	5d	> 4,971 mg a.e./kg diet = ST	-	> 1,742 mg a.e./kg diet = ST
Honeybee (*Apis mellifera*)	Contact 48h	> 100 µg a.e./bee = PNT	-	> 31 µg a.e./bee = PNT
Earthworm (*Eisenia foetida*)	14d	-	> 2,300 mg a.e./kg soil = PNT	> 1,550 mg a.e./kg soil = PNT

Legend: LC50 = median lethal concentration required to kill half the members of tested organisms after a specific time; EC50 = half maximal effective concentration which induces a response halfway between the baseline and maximum after a specific exposure time; LD50 = median lethal dose required to kill half the members of tested organisms after a specific time; MON = Monsanto; PNT = practically non-toxic; ST = slightly toxic; MT = moderately toxic.

In the aquatic environment, GIESY et al. (2000) found large differences in the sensitivity of organisms to GLY (factor 900). Crustaceans are often more sensitive to GBH than bacteria and protozoa (TSUI & CHU 2004; BRAUSCH & SMITH 2007). This finding may come unexpected since the pathway involving EPSPS is also present in bacteria, as mentioned before. The findings of the investigations are probably due to the more toxic substances of the tested GBH (cf. chapter 3.6.1) or GLY's mode of action is perhaps not restricted to the inhibition of EPSPS (cf. chapter 5.5). Similar to the terrestrial environment (i.e. in soil), GBH can induce community shifts in the aquatic environment. For example, PÉREZ et al. (2007) found strong community shifts in aquatic microbial communities towards GLY-tolerant species. PÉREZ et al. (2007) assessed the effect of Roundup® on freshwater microbial communities in Argentina using mesocosm experiments. However, the applied concentrations of 6 mg a.i./L and 12 mg a.i./L. exceeded the cited rate recommended for weed control (3.7 mg a.i./L; and even this is a relatively high concentration; cf. chapter 5.2) and were not compared with actual GLY measurements in standing waters. In this experiment, half-life values for GLY were 6 to 7 days. The abundance of picocyanobacteria (i.e. autotrophic picoplankton algae) increased 40 fold while all other microorganisms decreased. The increase of cyanobacteria is not surprising since these organisms are known to tolerate GLY either by producing more or a resistant form of the EPSP synthase (POWELL et al. 1991, 1992). The observed community shifts were consistent rather with a direct toxic effect of GLY than with an indirect effect mediated by phosphate release from the GLY molecule. Changes in the composition of the aquatic bacterial community could also be detected using molecular biology techniques (WIDENFALK et al. 2008). For additional studies on the impact of GLY and its formulations on freshwater microbial communities see VILLENEUVE et al. 2011: pp. 298-301 (freely available at http://www.intechopen.com/books/show/title/pesticides-formulations-effects-fate).

Interim conclusion

In summary, depending on concentration and species, GLY use can affect both terrestrial and aquatic organisms and communities. The active ingredient seems to be less toxic than the formulations used in practice. This is most likely an effect of the added substances in GBH that are often more toxic than GLY itself. The effects of both GLY and its formulations on amphibians, especially anuran larvae, are the main focus of this expert opinion.

3.4 The environmental fate of glyphosate

The German Advisory Council of the Environment (SRU 2008) stated that the highest risks of HR plants are the possible negative impacts on the environment. Hence the environmental fate of GLY as the most applied active ingredient of broad-spectrum herbicides worldwide is of particular importance, and also concerns possible exposure pathways to amphibians (see chapter 6).

Most of the studies concerning the environmental fate and the degradation of GLY date back to the 1970s-1990s. To date, opinions and findings on the potential negative effects of GLY on the environment (compared to the herbicides it replaces) are highly controversial. Below, we give an overview of the current state of knowledge about the fate of GLY in soil and water focussing on amphibian adult and larval habitats, respectively.

3.4.1 Fate in soil

ASPELIN (2003) concluded that the first registration of GLY in 1974 was an important date as he considered it an elementary systemic non-selective herbicide that quickly becomes inactivated in soil. The question may be asked what the meaning of ‘quick’ is here, and if there are any risks of GLY leaching into surface and ground waters. GLY could reach the soil by direct application and when washed from leaves by rainfall. It could also be released from crop residues and/or exuded from roots (e.g. NEUMANN et al. 2006). It is known that GLY is immobilized upon contact with soil and clay minerals due to the formation of surface complexes with metal ions (e.g. HANCE 1976; GLASS 1987; MORILLO et al. 2002). For example, GLY forms surface complexes on goethite, and the extent of the complexation is dependent on the ligand concentration in solution and the pH-value. Adsorption is favoured by lower pH-values since the adsorption of anions is coupled with a release of hydroxide (OH^-) ions (BARJA & DOS SANTOS AFONSO 2005). SPRANKLE et al. (1975) identified the phosphonic acid moiety as the main factor of adsorption. GLY competes with inorganic phosphate for soil binding sites (e.g. HILL 2001; GIMSING & BORGGAARD 2002a, b; BORGGAARD & GIMSING 2008). Thus, GLY can be reactivated by phosphate fertilisation as figured out by BOTT et al. 2011 (see chapter 7). In general, GLY is moderately persistent in soil and exhibits no pre-emergent activity compared with other pesticides (FRANZ et al. 1997). However, the intensity of adsorption as well as degradation of GLY differs with the type of soil and several other factors, such as temperature. For example, in a study by KOMOßA et al. (1992), half-life in soil was 217 days at low temperatures (2-15°C). In general, half-lives in soil ranged from 3 to 240 days (NEWTON et al. 1984; USDA 1984; USEPA 1990; GIESY et al. 2000; EUROPEAN COMMISSION 2002; BORGGAARD & GIMSING 2008). The primary route of mineralisation is through microbial degradation to AMPA (= aminomethylphosphonic acid) and then CO_2 (FRANZ et al. 1997; GIESY et al. 2000). SPRANKLE et al. (1975) reported rapid disappearance of GLY shortly after incubation and suggested that the adsorption of this herbicide to soil particles leads to it becoming unavailable to soil microorganisms. ARAÚJO et al. (2003) reported other findings in that GLY degradation is greatest between eight and 32 days of incubation. They also showed long-term effects of repeated GLY application. Compared to soils with no reported application, microbial activity increased in soils with reported previous application, showing that repeated application leads to increased microbial activity due to the utilisation of GLY as an available substrate. SINGH & WALKER (2006) overviewed the microbial degradation of organophosphates including GLY. Thereby, GLY seems to be co-degraded by some microorganisms and serves them as available P source. The ability to split the C-P bond is relatively widespread among microorganisms (PARKER et al. 1999). A possible explanation of the strongly fluctuating degradation rates of GLY in soils could be that a P deficiency would stimulate and a high P availability would slow down, respectively, the microbial degradation (see also chapter 7).

Due to the relatively strong sorption onto different soil minerals, GLY should be relatively non-leachable with a supposedly low tendency of run-off. However, run-off can occur, e.g. adsorbed to colloidal matter (SCHUETTE 1998). KJAER et al. (2003, 2009) found that in a Danish loamy soil GLY could leach through the root zone of plants into drainage water at ‘unacceptable’ concentrations (i.e. at average concentrations exceeding 0.1 µg/L), while the leaching risk of GLY in a coarse, sandy soil (also in Denmark) was negligible because of a matrix rich in aluminium and iron, providing good conditions for sorption and degradation. What is attributable for the leaching seems to be a combination of pronounced macropore flow occurring shortly after application and limited sorption and degradation capacity (KJAER et al. 2003, 2009). On the one hand, due to the relatively strong sorption onto soil minerals, the risk of leaching and run-off by GLY is limited compared to that of other pesticides. On the other hand, because soil structure and rainfall mainly determine GLY leaching and run-off, contamination of ground and surface waters is not impossible. While leaching is unlikely in non-structured sandy soils, this is more likely in structured soils with preferential flow in macropores, but only when heavy rainfalls follow GLY applications. Furthermore, GLY in drainage water can contaminate surface water, but not necessarily ground water because it may be sorbed and degraded in deeper soil layers (BORGGAARD & GIMSING 2008). For an overview of studies on GLY's run-off, see chapter 5.1.

In HR crop cultivations, Cry1 proteins, derived from an introduced Bt (= *Bacillus thuringiensis*) gene, in soil could arise from residues of previously cultivated GMO with insect resistant traits including single events and stacked events (see chapter 3.4; STEIN & RODRÍGUEZ-CEREZO 2009). It seems that the presence of Cry1Ac toxin does not impact on the mineralisation and bioavailability of GLY in soil (ACCINELLI et al. 2006). Last named authors took soil samples from a sandy loamy soil and from a sandy soil and obtained Cry1Ac toxin from a recombinant strain. In the laboratory, the presence of moderate concentrations (i.e. 0.25-1.0 µg/g soil) of Bt-derived Cry1Ac toxin had no appreciable impact on processes controlling the fate of GLY in both soil types (ACCINELLI et al. 2006). Specific studies on possible effects of other Cry1 proteins that were expressed by some GM crops are lacking.

Interim conclusion

BORGGAARD & GIMSING (2008), in their review on the fate of GLY in soil, advocated that the "... review has clearly shown that sorption, degradation and leaching of GLY can be very different from soil to soil, and much is still to be learnt about the fate of GLY in soils." The degree of sorption and degradation of GLY in soils is especially affected by following two factors: (i) soil structure and type and (ii) the amount of phosphate. Hence, it is impossible to unambiguously conclude the behaviour of GLY in soil. This has to be assessed site-specifically.

With regard to terrestrial amphibian life-stages, a possible risk would be contact with soil, where GBH have been applied. At first sight, this should represent a relatively low risk compared to that of other pesticides (e.g. BAKER 1985) because of the relatively strong sorption behaviour of GLY. However, (i) the degree of sorption depends on local conditions, (ii) specific studies on this kind of uptake in amphibians are largely missing (see BRÜHL et al. 2011) and (iii) although information on the fate of GLY is available, it is largely missing for the added substances.

Likewise, contamination of both terrestrial and aquatic amphibian habitats due to leaching and run-off can occur, depending on local conditions and especially when heavy rainfalls follow application. For detailed information on potential exposure pathways to amphibians, see chapter 6.

3.4.2 Fate in water

As figured out before, GLY can leach from soils to ground water and run-off from fields in surface waters under certain conditions. Following BORGGAARD & GIMSING (2008), our knowledge about the importance of subsurface leaching and surface run-off to water quality is scarce. Likewise, we know next to nothing about the amounts of wind-driven transportation of GLY, but also of other pesticides into surface waters (e.g. DAVIDSON 2004; TSUI & CHU 2008). Sometimes, standard drift rates are being calculated (for details see chapter 5.1). Of course, all possible water contaminations due to agriculture mainly depend on details of the application, especially the spraying height and the distance from the field margin. Besides illegal direct over-spraying of surface waters in agricultural practice, other application methods can directly contaminate water bodies. Mainly in forest management, aerial applications are conducted to kill broadleaf trees and favour the more marketable conifer trees (THOMPSON et al. 2004). Furthermore, aquatic habitats can be directly over-sprayed when aquatic weeds are combated with GBH labelled for aquatic use (BATTAGLIN et al. 2009). However, such application methods are not allowed in Germany.

GLY is highly soluble in water and relatively stable towards photodegradation and hydrolysis (KOLLMANN & SEGAWA 1995). Recently, ASSALIN et al. (2010) showed that the chemical breakdown of GLY requires harsh conditions with a maximum via ozonation at pH 10, which confirms that microbial degradation is the preferred route to mineralise GLY in nature. Under field conditions, GLY commonly dissipates from flowing surface waters, such as streams, through adsorption on sediments (e.g. FENG et al. 1990; SCHUETTE 1998) or alternatively is degraded to AMPA and finally CO_2 by microorganisms (BRIGHTWELL & MALIK 1978). The rate of dissipation from nonflowing waters, such as ponds, is mainly a function of the local conditions and should be considered site-specifically (GIESY et al. 2000). The degradation of GLY in water is generally slower as there are fewer microorganisms than in soil (GHASSEMI et al. 1981). First order half-life[7] ranged from 1.5 to 11.2 days in surface water ponds; in streams, residues were undetectable after 3 to 14 days (FENG et al. 1990; GOLDSBOROUGH et al. 1993; NEWTON et al. 1994). GIESY et al. (2000) estimated half-life values from 7 to 14 days. However, in laboratory studies using water from natural sources, half-life ranged from 35 to 63 days (USEPA 1986) and the EUROPEAN COMMISSION (2002) refers to half-life of GLY in water of 27 to 146 days.

Interim conclusion

The environmental fate of GLY differs from water body to water body as it is the case for different soil types. This is clearly demonstrated by the differing half-life values reported for GLY in water. GLY applications in agriculture can contaminate ground and surface waters due to leaching, run-off and drift. Direct over-spraying of water bodies in agricultural practice is illegal, at least in Germany. At least some of the selective herbicides, GLY should replace, have a worse effect on surface

[7] = half-life for a first order or unimolecular reaction, i.e. a reaction that depends on the concentration of only one reactant.

water quality than GLY due to worse environmental fate. For example, SHIPITALO & OWENS (2011) conducted a field experiment with conventional and HR maize and soybeans for three years. GLY amounts in surface run-off were significantly less than those of the active ingredients of the applied selective herbicides (alachlor, atrazine, linuron and metribuzin). Of course, this long-term experiment only refers to the local conditions (soil type etc.), but suggests a worse effect of herbicides other than GLY-based.

Amphibians that commonly reproduce in (small) water bodies and especially their embryos and larvae can come into contact with GLY and added substances that have found their way into surface waters. Published amounts of GLY in surface waters are listed in chapter 5.1. For detailed information on potential exposure pathways to amphibians, see chapter 6.

3.5 AMPA, the main degradation product

The majority of GLY is degraded by aerobic and anaerobic microorganisms to AMPA (= aminomethylphosphonic acid) and finally to carbon dioxide (GIESY et al. 2000). The adsorption patterns of AMPA and GLY to soil minerals such as goethite are similar (BARJA & DOS SANTOS AFONSO 2005), but AMPA adsorbs more strongly and thereby is less available for mineralisation through microorganisms (RUEPPEL et al. 1977). Hence, AMPA can accumulate in soil. Its half-life is normally greater than that of GLY and – depending on temperature and soil type – can vary between 78 and 857 days (GIESY et al. 2000; EUROPEAN COMMISSION 2002; BORGGAARD & GIMSING 2008). After two years, HENKELMANN (1992) recovered only 1% of the amount of GLY, but 45% of the amount of AMPA applied to soil samples. KJAER et al. (2003, 2009) detected long-term leaching of AMPA one and a half years after the application of a GBH. MAMY et al. (2010) also asserted the accumulation of AMPA in soil (for details on their study see chapter 3.4). They concluded: “the impacts of GT [= GLY tolerant] systems were lower than those of non-GT systems, but the accumulation in soils of one GLY metabolite (aminomethylphosphonic acid) questions the sustainability of GT systems”.

The half-life of AMPA in aquatic environments is comparable to that of GLY. Estimated from field studies, it ranges from 7 to 14 days (GIESY et al. 2000).

CAREY et al. (2008) did not include AMPA in their risk assessment of GLY because acute ecotoxicological studies on freshwater fishes, invertebrates and birds indicated that AMPA is not more toxic than its parent, GLY. However, because of its prolonged persistence and accumulation and resulting long-term leaching out of soils, AMPA is at least as relevant in environmental risk assessments as GLY itself.

3.6 Commercial formulations of glyphosate

Pesticide ingredients are divided into active and other, sometimes called ‘inert’, ingredients. Despite their name, ‘inert’ ingredients may be biologically or chemically active and are labelled inert only because of their function in the formulated product (COX & SURGAN 2006). In commercial end use GLY products, GLY as active ingredient is generally present in salt form with e.g. isopropylamine, potassium and ammonium as counterions (DILL et al. 2010). Because of its anionic nature, GLY does not penetrate the plant cuticle on its own (MANN et al. 2009), but requires a surfactant system that helps GLY to adhere to the surface and then penetrate into the leaf (MONSANTO 2005; DILL et al. 2010). Formulations are regarded as business secrets and usually details are not published (MERTENS 2011). The USEPA (1993) stated in its report for re-registration of GLY that: "...a toxic inert in GLY end use products necessitates the labelling of some products toxic to fish". Some commercial formulations have been found to have greater toxicity to aquatic and some terrestrial organisms than GLY due to the presence of a certain surfactant (DILL et al. 2010; see also Table 1). For example, BENACHOUR & SÉRALINI (2009) investigated the toxicity of four GBH on human cells in the laboratory. All tested formulations induced apoptosis and necrosis and caused total cell death, which the authors ascribed to the including surfactants.

In view of the future, reevaluation of GLY will be conducted complying only with the old and not the new requirements of the European Union (namely the more strictly European Plant Protection Products Regulation 1107/2009 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:EN:PDF) and the European Pesticides Framework Directive 2009/128/EC (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0071:0086:EN:PDF). With regard to the added substances, added components could be regarded according to the new requirements. ANTONIOU et al. (2011)[8] hold especially responsible the German Federal Office of Consumer Protection and Food Safety ('Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL) for playing down findings of serious risks of GLY and its formulations including endocrine disruption, carcinogenic effects, genotoxicity, neurotoxicity and especially teratogenic effects resulting in observed birth defects. ANTONIOU et al. (2011) especially refer to the study by PAGANELLI et al. (2010). The BVL answered the reproaches, for instance, with an official statement that questions the conclusions of PAGANELLI et al. (2010) (BVL 2010c; see http://www.powerbase.info/images/b/b8/BVL2010.comments.Paganelli.pdf). This once more suggests that the debate on the safety of GLY and GMO becomes controversial and perhaps sometimes steered by motivation other than science, especially when the natural environment is considered.

A total of 66 GBH are currently registered in Germany (BVL 2011), as are 50 in the USA (DILL et al. 2010). In the following points, some important formulations are briefly described.

- GLY was first marketed in the USA in the form of Monsanto's original Roundup® formulation (MON 2139) (MONSANTO 2005). Commercial forms of Roundup Original® normally contain about 31-41.6% active ingredient, i.e. GLY isopropylamine salt (MANN & BIDWELL 1999; REYLEA 2005b; DILL et al. 2010), about 15% surfactant, i.e. polyethoxylated tallowamine (POEA) (see chapter 3.3.1), water and other ingredients

[8] Note that ANTONIOU et al. (2011) is 'grey literature'.

(MERTENS 2011). In metric units, the proportion of active ingredient is approximately 360 g GLY a.e. per L or 480 g GLY isopropylamine salt per L (DILL et al. 2010). GIESY et al. (2000) conducted their ecological risk assessment on the original Roundup® formulation. Vision® is the name of a formulation often used in aerial applications in forest plantations. According to CHEN et al. (2004) and THOMPSON et al. (2004), Vision® is identical to Roundup Original®, i.e. it contains GLY isopropylamine salt (356 g a.e./L) and 15% POEA (MON0818).

- There are a number of different formulations with variations of the Roundup® brand name, which – because of their different surfactants – exhibit varying degrees of aquatic toxicity (DILL et al. 2010). For example, Roundup Original MAX® (Monsanto Company) contains 48.7% of active ingredient (i.e. GLY); the remaining 51.3% are other ingredients including an undisclosed surfactant (JONES et al. 2010). Roundup Biactive® (Monsanto Company) consists of 36% GLY isopropylamine and an undisclosed surfactant (MANN & BIDWELL 1999). Roundup WheaterMAX® (Monsanto Company) contains 48.8% GLY in the potassium salt form and an undisclosed surfactant (DILL et al. 2010). GLY is also sold as dry granular formulation, as Roundup WSD® particularly in South and Central America. Formulations containing more than one a.i. are commonly referred to as 'package mix formulations'. Typically, another type of herbicide (or herbicides) is added, e.g. GLY and 2,4-D (Landmaster®, Monsanto Company) or GLY, acetochlor and atrazine (Fieldmaster®, Monsanto Company). When preparing a formulation with more than one a.i., it will typically reduce the concentration in the final formulation for each a.i. (DILL et al. 2010). Package mix formulations are not to be confused with 'tank mixes[9]' which are combinations of two or more herbicides in the same spray tank prepared by the user.
- The original patents on the use of GLY and salts of GLY expired in 2000 (DUKE & POWLES 2008). Thereafter, for instance Touchdown® containing 39.5% of GLY in tetramethylsulfonium salt form was brought to market by Syngenta Company (Basel, Switzerland) (DILL et al. 2010). MANN & BIDWELL (1999) stated that Touchdown® consists of 48% GLY trimesium and alkylpolysaccharide and POEA as surfactants.
- Some formulations are designed and approved for application adjacent to water bodies (e.g., for non-indigenous plant control) such as Accord®, including the surfactant Timberline®90 (HOWE et al. 2004; BATTAGLIN et al. 2009), or Rodeo®, containing 53.8% GLY in the isopropylamine salt form, water and either X77® or R-11® as surfactant (PAVEGLIO et al. 1996; TRUMBO 2005). However, GIESY et al. (2000) stated that Rodeo® can be mixed with POEA as well. To the best of our knowledge, the toxicity to amphibians and other aquatic taxa of Timberline®90 has not yet been studied. In contrast, the ecotoxicological risk of nonylphenol polyethoxylate-based surfactants such as X77® or R-11® was assessed by BAKKE (2003). He assessed an over-spray and spill scenario and

stated that there is little risk to aquatic organisms living in a stream because there would be a short-term pulse of concentrated nonylphenol polyethoxylate-based surfactants moving downstream, mixing with water and being broken down or adsorbed into sediments. However, according to BAKKE (2003) under certain conditions the substances might have some toxic effects "In a stagnant small pond or stream reach, there could be effects seen on aquatic organisms (BAKKE 2003)." However, neither Accord® nor Rodeo® is used in agriculture.

The environmental fate in soil and water of those surfactants used in GBH are largely unexplored (for the surfactant POEA see chapter 3.3.1), but their potential impacts on aquatic organisms are controversially discussed. For example, SOLOMON & THOMPSON (2003) concluded from their ecological risk assessment that neither Rodeo® nor its usually added surfactants LI 700®, Induce® or X-77® pose a significant acute risk to aquatic organisms. The same is with PAVEGLIO et al. (1996). They investigated the environmental fate of Rodeo® and its surfactant X-77® at three estuary sites in Washington State (USA) (see also chapter 5.1) and concluded that "comparison of maximum concentrations for GLY in seawater from this study with acute toxicity values in the literature indicates that under worst-case conditions direct effects to aquatic organisms would not be likely." However, the major 'dissipations' of GLY were observed after the first high tide in their study. In contrast, general concerns about the more toxic effects of some GBH, especially to aquatic taxa, have been confirmed by other studies (e.g. HALLER & STOCKER 2003; TSUI & CHU 2003; BRAUSCH & SMITH 2007; BRAUSCH et al. 2007; see also next chapter) and COX & SURGAN (2006) advocate for a full assessment and monitoring of active and inert ingredients for pesticide registration under suitable regulatory acts.[10]

Interim conclusion

GLY is normally used in the form of GBH. Such formulations not only contain the active ingredient, but also different added substances, especially surfactants. Knowledge about the environmental fate of the active ingredient GLY itself and impacts on non-target organisms is one thing, but the effects of the added substances are largely unknown. Hence, different formulations bear different risks to non-target organisms, mainly depending on their surfactants, and, therefore, an unambiguous risk assessment for GLY use is impossible. Nevertheless, the general conclusion can be drawn that GLY itself is normally less toxic to animals than GBH and official testing for approval only considers the active ingredient (and one formulation as an example), but not the added substances.

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9 http://www.pesticides.gov.uk/applicant_guide.asp?id=1250

10 COX & SURGAN (2006) concluded: "Pesticide registration should require full assessment of formulations. Evaluations of pesticides under the National Environmental Policy Act, the Endangered Species Act and similar statutes should include impact assessment of formulations. Environmental monitoring for pesticides should include inert ingredients. To enable independent research and risk assessment, inert ingredients should be identified on product labels."

3.6.1 POEA – a special case

A common and highly effective surfactant in GBH is polyethoxylated tallowamine (POEA), a derivative of animal fat (JONES et al. 2010). Its fate in soil was investigated using three soils types (silt loam, silty clay loam and sandy loam) by MARVEL et al. (1974, unpublished study for Monsanto Company as cited in GIESY et al. 2000). In soil and also in natural waters containing sediments, POEA was primarily mineralised through microbial degradation (unpublished report for Monsanto Company by BANDHUN & FRAZIER 1974 cited in GIESY et al. 2000). Because few measurement data are available for POEA dissipation in both soil and water, GIESY et al. (2000) used more conservative estimates and, based on the measurements of MARVEL et al. and BANDHUN & FRAZIER, named half-life values ranging from 7 to 14 days in soil and from 21 to 42 days in water, respectively.[11] GIESY et al. (2000) estimated chronic exposure concentrations of POEA using a dissipation model. The highest chronic exposure concentration was 0.063 mg POEA/kg soil and 0.005 mg POEA/L water.

As with other surfactants, POEA is legally classified as an 'inert' ingredient. Some authors stated that GBH containing POEA pose slightly greater, but still only small risks for aquatic organisms compared with formulations containing other surfactants (e.g. SOLOMON & THOMPSON 2003). However, many other authors stated opposite conclusions: they found that POEA is the component that causes high mortality to aquatic organisms (e.g. MANN & BIDWELL 1999; HALLER & STOCKER 2003; HOWE et al. 2004; JONES et al. 2009). For example, TSUI & CHU (2003) conducted ecotoxicological tests with a bacterium, some microalgae, protozoa and crustaceans and named the following general toxicity order: POEA > Roundup® > GLY acid > GLY isopropylamine salt. This is also underlined by Table 1 which shows that the common GBH Roundup® including POEA is more toxic to several standard test organisms than the active ingredients alone. In case of aquatic organisms, Roundup® is about 40 to 50 times more toxic than GLY acid. BRAUSCH et al. (2007) examined the lethal and sublethal effects of three different POEA formulations (5:1, 10:1 and 15:1 average oxide:tallowamine) on *Daphnia magna*. All formulations inhibited growth at concentrations between 100 and 500 µg/L and the 10:1 POEA formulation was the most toxic with an $LC50_{48\text{-}h}$ of 97.0 µg/L.

When compared with estimated concentrations of POEA in water (0.005 mg/L = 5 µg/L; GIESY et al. 2000), the impact of POEA on daphnia should be negligible. At the same time GIESY et al. (2000) state that information on environmental POEA concentrations is very limited. Furthermore, the sensitivity of aquatic organisms to POEA is highly variable. For example, the Fairy shrimp *Thamnocephalus platyurus* is about 400 times more sensitive to POEA surfactants than *Daphnia magna* (e.g. $LC50_{48\text{-}h}$ 2.01 µg/L 15:1 formulation; BRAUSCH & SMITH 2007). In this

[11] GIESY et al. (2000) defined: "Dissipation of a substance from soil, in this review, is defined as loss by chemical breakdown or irreversible movement to other environmental compartments."

case, the highest environmental concentration estimated by GIESY et al. (2000) should have a catastrophic effect on a Fairy shrimp population.

With regard to amphibians, for instance, MANN & BIDWELL (1999) found that GBH with POEA are much more toxic to four Australian frog species than GLY on its own. This refers to tadpoles, metamorphs and adults. PERKINS et al. (2000) found that POEA was about 700 times more toxic than Rodeo® to embryonic *Xenopus laevis*, and several other authors arrived at similar conclusions (see chapters 5.4 to 5.6). Meanwhile, some companies even promote GBH with a 'frog-friendly' surfactant, i.e. not POEA (http://www.sipcam.com.au/files/Raze%20Flyer.pdf). However, the name and the proportion of the alternative surfactant are not given, which complicates a survey.

Interim conclusion

Both the environmental fate and environmentally concentrations of the popular surfactant POEA and largely unknown and the limited knowledge is only based on two industry studies. This is very important because scientific studies have shown that, when aquatic organisms including tadpoles were exposed to GBH, POEA is principally responsible for observed acute toxicity. Hence, with regard to the impact of GLY use on amphibians, particular attention should be paid to GBH with the surfactant POEA (detailed information are given in chapters 5.4 to 5.6).

3.7 Use of glyphosate in agricultures with genetically modified crops

As figured out before, GLY is mainly used in HR crop cultivation. GLY use exploded in the Americas since the admission of GM crops (see Figs. 1 and 2). According to DUKE & POWLES (2008), almost 90% of all GM crops worldwide are GLY-resistant to date and, in addition, the 'International Service for the Acquisition of Biotech Applications' (ISAAA 2010) stated that 83% of all GM crops worldwide display general herbicide resistance. It is worth mentioning that these numbers do not distinguish between GM crops with single transgenic traits and so called 'stacked events', where transgenes are stacked by conventional crossing of GM crops. STEIN & RODRÍGUEZ-CEREZO (2009) have shown the trend towards stacked events.

The debate about the potential advantages and impacts of HR crops related to the application of the complementary herbicide is controversial and, as already mentioned, perhaps sometimes steered by motivation other than science. The German Federal Office of Consumer Protection and Food Safety (BVL 2010b) stated that "unnecessary herbicide applications could be minimised because farmers can await the growth of weeds". This should result in less herbicide use (e.g. CHAMPION et al. 2003). For example in the cultivation of HR sugar beets, it is expected that the non-selective herbicide will be applied twice: the first application will usually be at the post-emergence stage while the second can take place up until the canopy has nearly closed. No additional application of selective herbicide should be necessary. In contrast, in the cultivation of conventional sugar beets a non-selective herbicide is usually applied at the pre-seeding or preemergence stage (i.e. no-tillage farming) and further two or three (or even up to five) applications of selective herbicides follow shortly after (GRAEF et al. 2010; SCHÜTTE & MERTENS 2010). However, long-term common use of any herbicide could cause resistances in weeds. In the case of GLY, this could lead to an additional re-use of 'conventional' selective herbicides besides the broad-spectrum herbicide. A periodic crop rotation could minimise the risk of the development of resistances, but the traits must be rotated as well as the crops, i.e. it has no effect if a GLY-resistant crop follows a GLY-resistant crop. The advantages of HR crops could lead to monotonous crop cultivation as has already happened in other parts of the world (BVL 2010b). Taking the cultivation of HR canola in the USA as an example, a reduced herbicide application could only be observed for the initial years, but over the long-term application rates were equal or even higher than in conventional cultivations (DALE et al. 2002; BENBROOK 2009, 2012). GRAEF et al. (2010) summarised potential positive and negative effects of the introduction of HR technology on the agro-environment (see Table 2). Following this, conducting a risk assessment is problematised as several experimental data sets on the potential adverse effects of the HR technology on the environment are lacking, but even without these missing data sets several adverse effects can be assumed (see also chapter 9).

Tab. 2: Some potential effects of the HR technology concerning amphibians and their habitats (modified after SRU 2008; KREMER & MEANS 2009; GRAEF et al. 2010).

Practice changes	**Chain of potential effects** (*positive*, negative)
Introduction of HR technology with exclusive use of GLY	Increased weed suppression → decreased agrobiodiversity → perhaps less food available Adverse effects on soil fungal communities and nutrient availability → increasing fertiliser use → contamination of nearby habitats and direct toxic effects on migrating and resting amphibians
Post-emergent spraying	*Less erosion due to more weed residues → less probability of pesticide run-off adsorbed on soil particles → less contamination of nearby habitats* Incompatibility with catch crops → increasing erosion → higher risk of run-off adsorbed on soil particles → higher contamination of nearby habitats Increased drift and run-off into nearby habitats due to increased spraying height, higher late season wind-speeds and increasing occurrence of heavy rainfalls → higher contamination of nearby habitats
Supposed periodical rotation of crops and traits	
Reduced herbicide (a.i.) amount and number of spray rounds	*Less negative impacts → less contamination and less toxic effects,* but only with a presumed equal toxicity of GLY and its formulations on amphibians than selective herbicides that are replaced (see chapter 5.4 to 5.6)
Supposed monotonous crop cultivation without rotation	
Development of GLY-resistance and control of the increasing GLY-resistant weeds	Increased use of GBH and re-use of selective herbicides → equal or increased herbicide (a.i.) amount, number of spray rounds → equal or higher contamination and toxic effects than in conventional agriculture

MAMY et al. (2008) assessed the environmental impact of herbicides applied during the cultivation of HR and non-HR crops. They modelled the environmental fate of different herbicides including GLY. By doing so, they parameterised their model with laboratory data and those from field sites in France where both HR and non-HR crops had been cultivated for several years. All field applications represent normal-use scenarios. Soil samples of different depths were taken after application, in autumn, winter and the following spring. Dissipation rates of the herbicides and concentrations of AMPA were analysed. With the results, the movement of the herbicides in the unsaturated soil layer was simulated using a 1-D pesticide root zone model. Persistence increased as follows: metazachlor < GLY < trifluralin, i.e. GLY was not the least persistent of the three herbicides as previously supposed. AMPA and trifluralin had the largest vertical mobility and were found in the deep soil layer. Hence, the authors came to the conclusion that the replacement of both metazachlor and trifluralin with GLY might not decrease the environmental impact. MAMY et al. (2010) came to the conclusion that the impact of GLY-resistant systems on ground water, air and human health would be lower than those they replace. In this study, no field data were sampled. The model was mainly parameterised with the field data collected in 2008 (MAMY et al. 2008). The overall impact of an HR crop cultivation mainly depended on the actual rate and frequency of GLY application in the model. It was highest in HR maize monocultures and lowest in a combined HR canola/non-HR sugar beet crop cultivation. However, MAMY et al. (2010) again questioned the sustainability of HR crop systems because of the observed high accumulation of GLY's major metabolite AMPA in soil (as already mentioned in chapter 3.2). In addition, they only modelled one GLY application but several are probable (and it is also probable that other herbicides would be applied).

In Germany, procedures for monitoring the potential effects of GMO on non-target organisms have been and continue to be developed (SEITZ et al. 2010). Guidelines for an assessment of potential direct and indirect effects of GMO (mainly HR crops) on amphibians via a monitoring approach are provided in BÖLL et al. (in press) and guideline 4333 from the VDI (in press).

There are more potentially adverse effects of the introduction of HR technology on the environment (e.g. hybridisation with wild plants) and also on crops (e.g. hybridisation with non-HR crops, increasing plant diseases, see also chapter 7) than presented in this chapter. In the following, we only consider those potentially affecting amphibians and their habitats. The acute and chronic toxicity of GLY and its formulations on amphibians as well as interactions with other stressors is presented in detail in chapters 5.4 to 5.6. For a more detailed overview on the overall impacts of the introduction of HR technology, using GM sugar beets as the example, see GRAEF et al. (2010; http://www.livingreviews.org/lrlr-2010-3) and SCHÜTTE & MERTENS (2010; http://www.bfn.de/fileadmin/MDB/documents/service/Skript%20277.pdf).

3.8 Use of glyphosate in conventional agricultures and for other purposes

GBH are not only used in the complementary system with HR crops but also in conventional cropping systems, mainly in no-tillage farming. This is of particular importance for Germany where GM crop cultivation is still negligible. However, GLY application is only possible before the crops emerge and after the harvest[12] (HENKELMANN 2001; SCHÜTTE & MERTENS 2010). In some cultivations (e.g. winter canola), GBH are commonly used as desiccant to speed up the harvest, i.e. the crops are sprayed, die and quickly dry off (for German constrictions due to tallowamine surfactants in some formulations, see also http://www.lfl.bayern.de/ips/unkraut/39479/linkurl_0_2.pdf). RAUBUCH & SCHIEFERSTEIN (2002) found that the agricultural use of GLY had increased in German conventional cultures from 1,093 tons in 1993 to 2,745 tons in 2000. This information conforms to the amounts named by HOMMEL & PALLUTT (2003). The 'Neptun' program was a survey on the application of chemical pesticides in agricultural practice in Germany in the year 2000 (ROßBERG et al. 2002). Amongst all herbicides used in the cultivation of winter rye, GLY ranked 12 out of 20 (1.8%). It ranked 13 out of 25 herbicides applied in the cultivation of winter wheat (3.0%), 5 out of 14 in the cultivation of winter barley (5.0%), 16 out of 20 in the cultivation of triticale (1.5%), 13 out of 21 in the cultivation of summer barley (3.2%), 12 out of 12 in the cultivation of oats, 7 out of 10 in the cultivation of potatoes (2.1%), 10 out of 10 in the cultivation of canola, 8 out of 10 in the cultivation of sugar beets (2.0%) and 11 out of 13 in the cultivation of maize (2.1%). As a conclusion of the 'Neptun' survey, before 2000 the use of GLY in German conventional agriculture was circumstantial compared to the use of the other herbicides. However, the 'Neptun' survey was completely based only on voluntary information (ROßBERG et al. 2002). Furthermore, the GLY applications steadily increased from the 1980s until the last decade (RAUBUCH & SCHIEFERSTEIN 2002; HOMMEL & PALLUTT 2003), and from the years 2004 to 2009, GLY has been amongst the highest quantities of sales among all agricultural pesticides traded in the country (i.e. more than 1,000 tons per year; BVL 2005, 2006, 2007, 2008, 2009, 2010a). In the years 2004, 2005, 2008 and 2009, isoproturon was the only herbicide besides GLY in this group (BVL 2005, 2006, 2009, 2010a). This means that in Germany, a country in which GM crops are yet negligible, the use of GLY has also increased in recent years. As mentioned before, this appears to be mainly related to the increasing no-till farming performed in Germany, partly funded by some German states (RAUBUCH & SCHIEFERSTEIN 2002).

Besides use in cultivation of conventional crops, GLY is also used in vegetable gardening, winegrowing, fruit-growing, cultivation of ornamental plants, on grasslands and in forests, on non-cultivated areas as well as for private use in gardens (BVL 2011). Application on non-cultivated

[12] Bands spraying is occasionally possible, but only until the canopy has closed.

areas includes railways and highways, and the latter requires at least a special permit because run-off in nearby surface waters is likely.

4. Methods

4.1 Literature review

We used ‘Web of Knowledge’ (Thomson Reuters®) and ‘Google Scholar®’ to search for published scientific articles using following key words: “glyphosate*”, “glyphosate* + amphibian*”, “Roundup”, “Roundup + amphibian*” and “amphibian decline*” for the time period October 2010 to June 2011. In total, 204 relevant articles were found. In addition, we examined the cited references in the relevant studies and obtained unpublished data from ‘grey literature’, largely such distributed via national authorities (e.g. expert opinions). Summarised, 326 documents dealing with the environmental fate, concentrations and impacts of GLY and GBH on non-target organisms were exploited in this expert opinion.

4.2 Macroecological approach

In addition to the literature review, we chose a macroecological approach to study potential correlations between long-term amphibian population trends and agricultural factors including pesticide and fertiliser consumption, land use and its change. It is important to mention that only correlative allusions can be made with such an approach. Causal allusions require more targeted and comprehensive laboratory, mesocosm and field studies (cf. chapters 5.4 to 5.6). Furthermore, as already mentioned, amphibian decline and extinction is a complex field with several factors and interactions at work (see chapter 2 and Fig. 3). Hence, these results will only concern the influence of the available data on the considered amphibian populations. We restricted our analyses to the USA and Germany due to the lack of data in other target regions of this expert opinion. It is mentionable that also for these two regions only ‘raw’ data were available, thus limiting prospective conclusions.

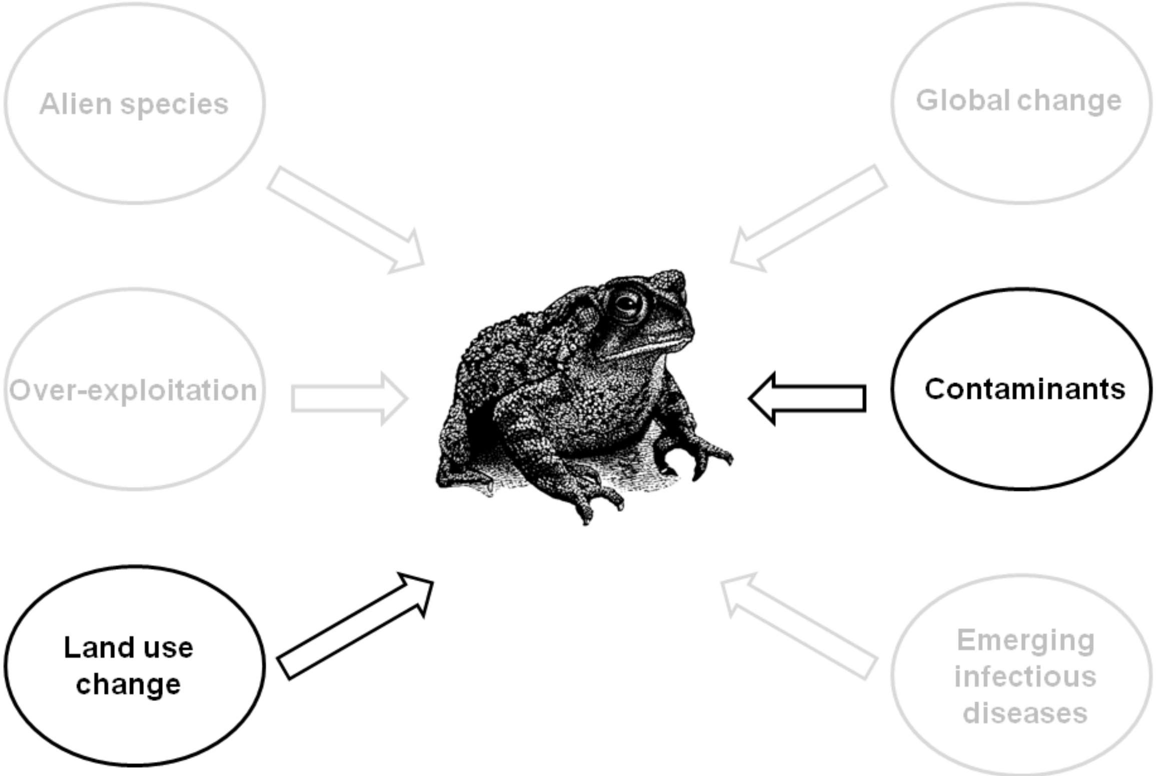

Fig. 3: Agricultural stressors, which were considered in the analytical approach.
Both stressors can only be related to two out of six groups, which likely play a role in amphibian decline (according to COLLINS & STORFER 2003; cf. chapter 2). Part of the figure is based on drawings from http://commons.wikimedia.org

4.2.1 Data sets

Amphibian populations and species

When studying amphibian population dynamics and trends (both natural and human-driven), it is crucial to put the active factors into a context with overall population dynamics (i.e. natural population fluctuations) of particular species (HOULAHAN et al. 2000; BIEK et al. 2002). That is why we only used amphibian population time-series sampled over at least three up to 20 years. It is mandatory to consider that amphibians have complex life cycles in which density-dependent regulation may occur at the larval, the juvenile and the adult stage (e.g. MEYER et al. 1998), often resulting into large natural population fluctuations. We tried to deal with this problem by using a large amount of population data.

For Germany, we received count data obtained with the help of amphibian drift fences attended by various volunteers (see Acknowledgements) and managed by the German 'Nature and Biodiversity Conservation Union' (NABU). The main problem of count data is imperfect detection probability (e.g. SCHMIDT 2004a). To account for this, we only used count data of European 'explosive' breeders (listed in Table 3). With regard to the length of the breeding season, most

European amphibian species can be regarded either ‘explosive’ or ‘prolonged’ breeders (ARAK 1983; WELLS 2007). When employing drift fences within the short period the adults of ‘explosively’ breeding amphibian species migrate to the reproduction sites, we suppose that a large part of the adult population could be accessed. Furthermore, we separated sites with strongly differing length of the drift fences out, also information that appeared implausible. However, even when the lengths of the drift fences were similar, differences in sampling time, effort and duration are probably leading to imperfect detections in most cases. Therefore and because natural population sizes strongly differed at sites, we only considered the growth rates of an amphibian population and not its total number of individuals, i.e. time series started with ‘1’ and continued with ‘< 1’, when a negative trend was observed, or ’> 1’ when a positive trend was observed, respectively.

On the one hand, North American data are based on calling surveys. Data sets were taken from the ‘North American Amphibian Monitoring Program’ of the U.S. Geological Survey (for details see http://www.pwrc.usgs.gov/naamp/). Here, male anuran choruses are classified with the help of three indices[13] in ascending order (low/middle/high). Data of the monitoring program were relatively recent (2001-2010) and included the time period with a rapid increase in GLY consumption, hence.

On the other hand, we thankworthy got access to the collection of HOULAHAN et al. (2000), certainly the most complete compilation of amphibian population size estimates available up to 1998. However, because of different methods (calling indices/growth rates), we could not merge the data of the U.S. Geological Survey and of HOULAHAN et al. (2000) and, therefore, analysed them separately.

Many amphibian species could be affected by agrarian practices like herbicide applications during their annual migration to breeding sites, summer and winter habitats etc. Other amphibians with habitat away of agricultural areas are supposed to be affected by pesticide drift via wind (e.g. DAVIDSON 2004; FELLERS et al. 2004). Intensively used agricultural land plays a minor role as habitat for most amphibians. However, amphibian populations can be found in extensive used structures like hedgerows or fallows and reproduce in small ponds, drainage ditches as well as ephemeral water bodies embedded within agrarian areas (cf. chapter 6). As amphibian species relevant for analysis, we only considered those fulfilling two criteria: (i) their reproduction sites and/or terrestrial habitats can be situated within or nearby agricultural areas which makes them potentially affected by GLY applications; (ii) data of at least 30 populations with a ‘starting number’ (i.e. counts/estimates in the first survey year) of at least 15 individuals were available so that sample size was sufficient for analysis. Furthermore, time-series had to be sampled over at least three years. As a result, although several other species could be more related to agricultural areas (like the Common spadefoot toad, *Pelobates fuscus*), we more focussed on species which are common (Table 3). Because 30 or more populations of not one North American amphibian species

[13] Calling Index 1 = individuals can be counted; there is space between calls; Calling Index 2 = calls of individuals can be distinguished but there is some overlapping of calls; Calling Index 3 = full chorus, calls

were available from 1990-1998, we had to summarise all US amphibian populations of the database by HOULAHAN et al. (2000) (see Appendix 1). For it, we calculated the growth rates of every population and merged them to one response variable (SPECIES).

Tab. 3: Selected anuran species for analysis.

Species – family		**Study duration**
Germany	**No. of populations**	
Common toad (*Bufo bufo*) – Bufonidae	262	1991-2009
Common frog (*Rana temporaria*) – Ranidae	80	1991-2009
Moor frog (*Rana arvalis*) – Ranidae	30	1991-2009
USA	**No. of surveys**	
Northern cricket frog (*Acris crepitans*) – Hylidae	1,419	2001-2010
American toad (*Anaxyrus americanus*) – Bufonidae	2,014	2001-2010
Northern leopard frog (*Lithobates pipiens*) – Ranidae	148	2001-2010

BLAB (1978) named the Common toad (*Bufo bufo*) a "euryoecious forest species" meaning this toad prefers forests, but can also be found in a multitude of other habitats. Large open areas are mainly avoided, but in forested landscapes toads also inhabit meadows, parks, gardens and also extensive used structures in agricultural areas (e.g. hedgerows). Mainly vegetated and permanent breeding ponds are used but also, for instance, drainage ditches (SOWIG & LAUFER 2007; AGASYAN et al. 2008). Also the Common frog (*Rana temporaria*) can be classified as a euryoecious amphibian. Although it avoids large open and intensively used fields, it can occasionally be found in extensively used structures within agricultural areas. Drainage ditches can serve as dispersal corridor (WOLSBECK et al. 2007). The Common frog only occasionally breeds directly within intensively used agrarian areas (e.g. LOMAN & LARDNER 2006) and has declined in areas dominated by intensive agriculture mainly in the 1950s and 1960s due to the intensification of agriculture (SCHLÜPMANN & GÜNTHER 1996). However, ditches and small ponds embedded within agrarian landscapes can be exploited for reproduction, besides a wide array of other breeding ponds (e.g. SCHNEEWEISS 1996). In general, the Moor frog (*Rana arvalis*) prefers areas with a high ground water level like marshes. It is more abundant in the northern and north-eastern parts of Germany (LAUFER & PIEH 2007). Adults can be found on fields in summer, also when slightly dry, but especially when water logging areas are present (e.g.

are constant, continuous and overlapping.

DÜRR et al. 1999). Reproduction takes place in different water bodies including flooded areas and drainage ditches (LAUFER & PIEH 2007).

The Northern cricket frog (*Acris crepitans*) uses a variety of mainly open habitats and reproduces in several types of water bodies (HAMMERSON et al. 2004a). For example, five out of nine breeding ponds of this species studied by BEASLY et al. (2005) were surrounded by agricultural land use. The American toad (*Anaxyrus americanus*) is a highly adaptive species and can be found in various habitats including fields and agricultural areas (e.g. KLEMENS 1993; HAMMERSON 2004). As pointed out by GREEN & PAULER (1987), in West Virginia, these toads are even more frequent in open pastures, gardens and agricultural areas than in forests. Both permanent and ephemeral ponds are accepted for reproduction (HAMMERSON 2004). Adults of the Northern leopard frog (*Lithobates pipiens*) can often be found during the summer on fields. Reproduction commonly takes places in permanent water bodies (HAMMERSON et al. 2004b).

Pesticide usage

“Time-series of amphibian populations are of limited value if we try to understand only the mechanisms leading to declines we observe at present” (MEYER et al. 1998). That is why for both Germany and the USA we looked for historical data on the usage of GLY and other herbicides. Also information on the main herbicidal groups and total usage of herbicides were considered in our analysis. Furthermore, main ingredient groups and total consumption of insecticides, fungicides, bactericides and plant growth regulators were considered for our study regions.

For the USA, we used reports on market estimates on GLY use from the USEPA (ASPELIN 1997; ASPELIN & GRUBE 1999; DONALDSON et al. 2002; KIELY et al. 2004; GRUBE et al. 2011), published in a two-year-rhythm, as a proxy. We took the same references to obtain information on other herbicides. Atrazine, metolachlor, metolachlor-s, acetochlor and 2,4-D were considered, as these herbicides were among the top five in US agricultural use between 1987 and 2007 (ASPELIN 1997; ASPELIN & GRUBE 1999; DONALDSON et al. 2002; KIELY et al. 2004; GRUBE et al. 2011). These references were also used for information on cumulative use of herbicides, insecticides and fungicides. GLY was also one of the agricultural pesticides with the highest quantities of sale in Germany from the years 2004 to 2009 (i.e. over 1,000 tons per year; BVL 2005, 2006, 2007, 2008, 2009, 2010a). However, no details on the application of GLY or other herbicides were available for Germany. Hence, general data on pesticide usage for Germany were obtained via the statistical service of the FAO (2011). The FAO (2011) discriminates pesticides into main groups of ingredients (cf. Table 4). It needs to be added that GLY is listed here under ‘other herbicides’ (OHE). Also for information on cumulative usage of herbicides, insecticides and fungicides this reference was used.

Additional variables

Fertiliser usage (i.e. inorganic and organic fertilisers excluding farm fertilisers as slurry, dung and sludge) for both regions should be considered as another expected main factor of negative impact of agriculture on amphibians in general. For example, fertilisers could have negative impacts on amphibians like chemical burns or destruction of breeding ponds due to proceeding eutrophication (for an overview see MANN et al. 2009). For the USA, information was obtained via the statistical service of the FAO (2011) available from 1990-2007 only. For Germany, information was available from 1990-2008 (FAO 2011).

For the USA, we obtained information on the land use change from conventional agriculture to agriculture with GM crops adopted from the USDA (2010). For amphibian populations from Germany, detailed coordinates were available, thus accounting for a link to actual land use and land use change in a GIS-based approach related to studied populations. For this purpose, we created 1 km buffers with ArcGIS 9.3® (ESRI) around amphibian records and addressed the land use within them. There is information on observed higher dispersal power of the considered 'explosive breeders' (e.g. JEHLE & SINSCH 2007). However, we considered 1 km as an average migration distance for these species. Land use data were those of the European CORINE land cover project (http://www.corine.dfd.dlr.de/intro_de.html) and were available for the years 1990, 2000 and 2006. We plausibly grouped some CORINE variables (see Tables 4 and 5 for an overview of potential impact factors used in the analysis).

Tab. 4: Definition of agrochemical variables used in analysis and their availability.

Variable	Abbre-viation	Country	Period of estimated consumption of active ingredient
USE OF SELECTED HERBICIDES			
GLY	GLY	USA	2001-2007
Atrazine	ATR		
Metolachlor	MET		
Metolachlor-S	MES		
Acetochlor	ACE		
2,4-D	24D		
USE OF PESTICIDES ACCORDING TO MAIN INGREDIENT GROUPS			
Herbicides			
Phenoxy hormone products	HOR	DE	1990-2009
Triazines	TRI		
Amides	AMI		
Carbamate herbicides	CAR		
Dinitroanilines	DIN		
Urea derivates	URE		
Sulfonyl ureas	SUL		
Bipiridils	BIP		
Other herbicides (including GLY)	OHE		
Insecticides			
Chlorinated hydrocarbons	CHL	DE	1990-2009
Organophosphates	ORG		
Carbamate insecticides	CAI		
Pyrethroids	PYR		
Botanical and biological products	BOT		
Other insecticides	OIN		
Fungicides & bactericides			
Inorganics	INO	DE	1990-2009
Dithiocarbamates	DIT		
Benzimidazoles	BEN		
Diazoles and triazoles	DIA		
Morpholines and diazines	MOR		
Other fungicides and bactericides	OFU		
TOTAL USE OF PESTICIDES			
Total herbicide use	HER_TOT	USA	1996-2007
Total herbicide use	HER_TOT	DE	1990-2009
Total insecticide use	INS_TOT	USA	1996-2007
Total insecticide use	INS_TOT	DE	1990-2009
Total fungicide and bactericide use	FUN_TOT	USA	1996-2007
Total fungicide and bactericide use	FUN_TOT	DE	1990-2009
Total plant growth regulator use	PLA_TOT	DE	1990-2009
TOTAL USE OF FERTILISERS[14]			
Total use of inorganic and organic fertilisers	FERT_TOT	USA	1990-2007
Total use of inorganic and organic fertilisers	FERT_TOT	DE	1990-2008

[14] Excluding farm fertilisers as slurry, dung and sludge.

Tab. 5: Definition of land use variables used in analysis and their availability.

Variable	Abbreviation	Country	Proportion of cultivation
Total agricultural area	AGR_TOT	USA	Total area, 1990-2009
Cultivation of HR soy	HR_SOY	USA	1996-2009
Cultivation of HR cotton	HR_COT		
Cultivation of HR maize	HR_MAI		
Cultivation of BT cotton	BT_COT		
Cultivation of BT maize	BT_MAI		
Urban areas (CORINE variables 111+112)	URB	DE	in a 1 km buffer; 1990, 2000, 2006
Industrial units (CORINE variables 121+122+123)	IND		
Mineral extraction sites and ruderal areas (CORINE variables 131+132+133)	MIN		
Parks, green urban areas and sport fields (CORINE variables 141+142)	PAR		
Acres (CORINE variable 211)	ACR		
Vineyards (CORINE variable 221)	VIN		
Fruit trees and berry cultivations (CORINE variable 222)	FTR		
Pastures (CORINE variable 231)	PAS		
Complex cultivation patterns, i.e. small-area change in cultivation (CORINE variable 242)	CUL		
Acres with larger parts of natural vegetation (CORINE variable 243)	ACN		
Forests (CORINE variables 311+313)	FOR		
Coniferous forest, i.e. mainly monocultures of *Picea abies* or *Pinus sylvestris* (CORINE variable 312)	CON		
Natural grasslands (CORINE variable 321)	GRAS		
Moors and peat bogs (CORINE variables 322+412)	MOO		
Transitional woodland-shrubs (CORINE variable 324)	SHR		
Inland marshes (CORINE variable 411)	MAR		
Water courses, water bodies and sea (CORINE variables 511+512+521+523)	WAT		

Consideration of the variables

We suppose that use of agrochemicals have delayed effects on amphibians, i.e. the consumption in year X can affect an amphibian population in this year, but effects can be only observed later. Hence, time series for possible impact factors such as pesticide and fertiliser consumptions started one, three and four years prior to time series for amphibian populations. Samplings and call surveys always took place in or at least nearby reproductive sites. Hence, negative impacts of historical consumptions should result in less mature adults to return to the reproductive sites. Therefore, when agrochemical consumptions are considered in the year prior to time series of amphibians, direct impacts on (migrating) adults should be visible. For example, MANN & BIDWELL (1999), RELYEA (2005b) and BERNAL et al. (2009b) observed high mortality of terrestrial amphibians when directly exposed to GBH.

It is difficult to name a specific time until sexual maturity of most amphibian species.

Different authors suggested that the attainment of sexual maturity in amphibians is dependent on reaching a certain body size rather than a specific age (see HALLIDAY & VERELL 1988; READING 1991 and references therein). Nevertheless, we assumed an average age of two to three years to reach sexual maturity for all considered species[15]. Hence, when considering consumptions three and four years prior respectively to amphibian time series, impacts on larvae, metamorphose rate and the survival of juveniles (e.g. due to reduced fitness) should become revealed. For simplification, time of consideration of the predictor set is given in parentheses (X years prior) in the following.

4.2.2 Statistical methods

We detected the predictors which significantly explain variance using 'Boosted Regression Trees' (BRT) (FRIEDMAN 2001). This method has been successfully applied in ecological approaches (e.g. LEATHWICK et al. 2006; ELITH et al. 2008). 'Traditional' regression methods like Generalised Linear Models (GLMs) or Generalised Additive Models (GAMs) produce a single 'best' model that describes the relationships between a response variable and a set of predictors. In contrast, BRT use the technique of boosting to combine large numbers of relatively simple models. This stochastic component improves predictive performance and reduces the variance of the final model. Each model consists of a simple regression tree. Trees were progressively added while re-weighting the data to emphasize cases poorly predicted previously. Decision trees were fitted by collapsing the weakest links identified through a cross-validation implementation. Therefore, the predictions of all models are combined in the final model and interactions between variables were considered (for further information on BRT, see ELITH et al. 2008). For analysis the software R (R DEVELOPMENT CORE TEAM 2009) was employed using the 'gbm' library (RIDGEWAY 2007) plus an additional code written by ELITH et al. (2008). To account for normal distribution, the responsible variables consisting of growth rates had to be logarithmised first. Furthermore, we used the factorial function for the variable 'year' ('factor_year') in each calculation. We started calculations with a learning rate of 0.001, a tree complexity of 5 and a maximum number of 5,000 trees. Predictor sets of all final models eventually were simplified, i.e. all predictors having only minimal effects on prediction were dropped and only the most influential variables remained.

[15] This is not true for at least female Common toads which may need six to seven years to reach sexual maturity (SOWIG & LAUFER 2007). However, the majority of adults in most Common toad populations are males and females sometimes skip breeding in some years and do not migrate to the reproduction ponds (LOMAN & MADSEN 2010).

5. Results

5.1 Results of the macroecological approach

Because the BRT method considers all interactions in the predictor set due to combining the predictions of all calculated models in the final model, the effect of a certain predictor can only be interpreted by plotting the fitted functions. Hence, some final predictors do not show a clear significant trend (cf. Fig. 4) as normally observed by the remaining predictors selected by ‘traditional’ methods like stepwise regression. Anyhow, the variables selected by BRT have an important effect on prediction.

As already mentioned, agrochemical consumptions were considered one year prior to time series of amphibians to indicate possible effects on (migrating) adults and three and four years prior respectively to indicate possible effects on larvae, metamorphs and juveniles.

5.1.1 Amphibian populations from Germany

Tables 6 and 7 show the results of the BRT calculations for three amphibian species from Germany performed with 26 agrochemical variables and 17 land use variables. For definitions of variables see Tables 4 and 5 in chapter 4.2. Information on residual deviance and correlation indicates good fits for all final models (the residual deviance has to be as low as possible and a correlation coefficient of 1.0 would represent a perfect match; cf. Table 6).

Tab. 6 Final models for amphibian populations from Germany.

Model	Mean residual deviance	Cross validated residual deviance (± SE)	Training data correlation	Cross validated correlation (± SE)
Common toad (*Bufo bufo*) (1 year prior)	0.01	0.03 ± 0.00	0.90	0.74 ± 0.01
Common toad (*Bufo bufo*) (3 years prior)	0.04	0.04 ± 0.00	0.70	0.63 ± 0.02
Common toad (*Bufo bufo*) (4 years prior)	0.04	0.04 ± 0.00	0.71	0.63 ± 0.01
Common frog (*Rana temporaria*) (1 year prior)	0.04	0.06 ± 0.01	0.75	0.55 ± 0.03
Common frog (*Rana temporaria*) (3 years prior)	0.04	0.06 ± 0.00	0.75	0.57 ± 0.04
Common frog (*Rana temporaria*) (4 years prior)	0.04	0.06 ± 0.01	0.75	0.59 ± 0.04
Moor frog (*Rana arvalis*) (1 year prior)	0.03	0.06 ± 0.01	0.86	0.64 ± 0.05
Moor frog (*Rana arvalis*) (3 years prior)	0.03	0.07 ± 0.01	0.86	0.61 ± 0.06
Moor frog (*Rana arvalis*) (4 years prior)	0.03	0.07 ± 0.01	0.86	0.62 ± 0.07

Tab. 7: Most important predictors out of 43 variables tested for effects on amphibian populations from Germany.

Response variable	Consumption of agrochemicals	Explanatory land use variable (% of explained variance)	Supposed effect
Common toad	One year prior	ACR (14.1)	-
(*Bufo bufo*)	(impact on adults)	factor_year (13)	-
		CON (12.3)	Positive
		URB (11.5)	Negative
		CUL (9.9)	Negative
		FOR (8.7)	-
		PAS (7.5)	-
		MIN (4.0)	Positive
		VIN (2.4)	-
		PAR (2.4)	-
		SHR (2.2)	-
		ACN (2.1)	-
	Three years prior	CON (16.1)	Positive
	(impact on juvenile life-	ACR (13.7)	-
	stages)	CUL (12.6)	-
		URB (11.9)	Negative
	Four years prior	CON (16.0)	Positive
	(impact on juvenile life-	ACR (13.6)	-
	stages)	CUL (12.6)	-
		URB (11.9)	Negative
Common frog	One year prior	factor_year (21.8)	Negative
(*Rana temporaria*)	(impact on adults)	ACR (15.6)	Positive
		FOR (15.5)	Negative
	Three years prior	factor_year (17.4)	Negative
	(impact on juvenile life-	FOR (16.4)	Negative
	stages)	ACR (15.8)	Positive
		URB (13)	Negative
		VIN (12.5)	Negative
	Four years prior	ACR (15.9)	Positive
	(impact on juvenile life-	FOR (15.9)	Negative
	stages)	URB (13.4)	Negative
Moor frog	One year prior	factor_year (28.2)	-
(*Rana arvalis*)	(impact on adults)	ACN (21.4)	Positive

	ACR (17.4)	-
Three years prior (impact on juvenile life-stages)	factor_year (24.3)	-
	ACN (21.3)	Positive
	ACR (17.3)	-
Four years prior (impact on juvenile life-stages)	factor_year (23.5)	-
	ACN (21.4)	Positive
	ACR (17.3)	-

Legend: ACR = Acres; CON = Coniferous forest; URB = Urban areas; CUL = Complex cultivation patterns; FOR = Forests; PAS = Pastures; MIN = Mineral extraction sites and ruderal areas; VIN = Vineyards; PAR = Parks, green urban areas and sport fields; SHR = Transitional woodland-shrubs; CAN = Acres with larger parts of natural vegetation

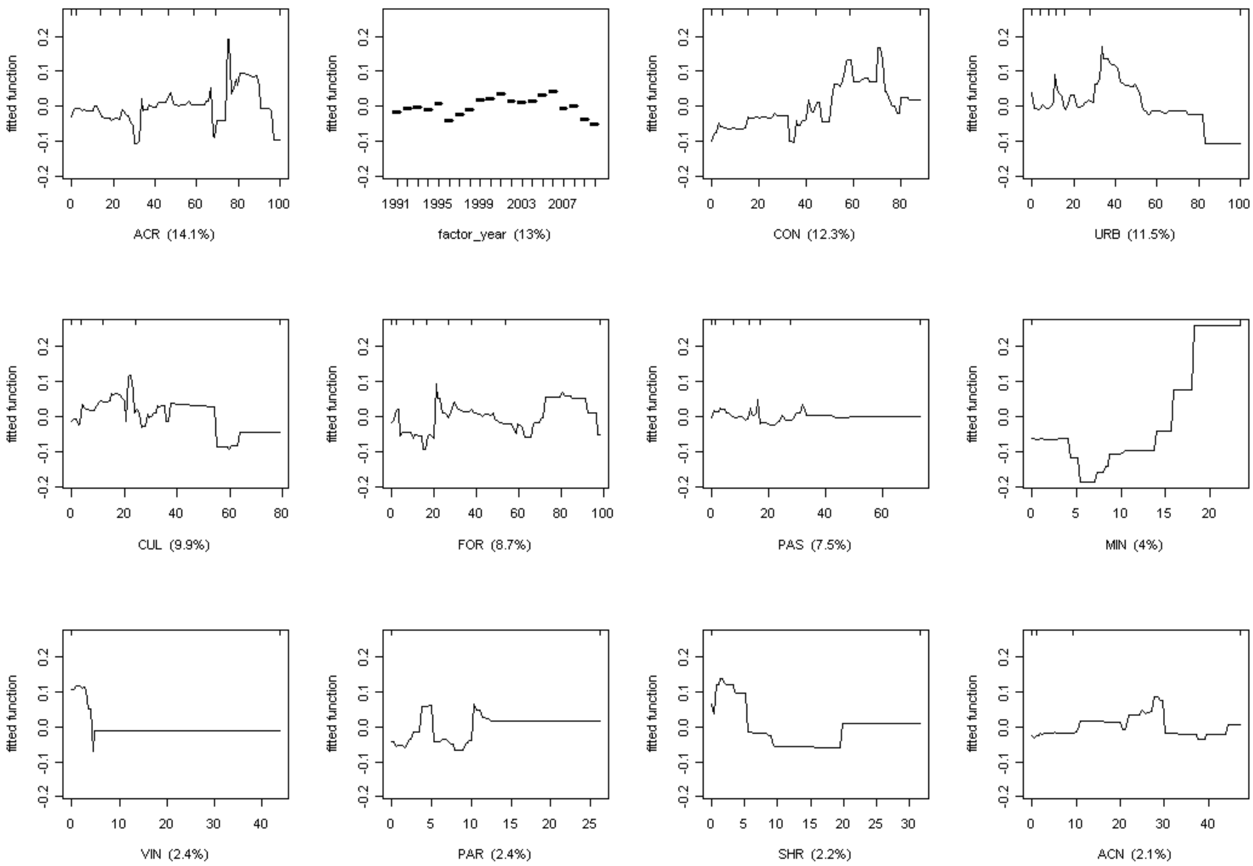

Fig. 4: Fitted functions of the most important predictors affecting adult Common toads (*Bufo bufo*). Note that positive fitted functions suggest that species respond favorably and low values suggest the opposite.

After simplifying the final model, twelve variables are left that have effects on adult Common toads (*Bufo bufo*) (Table 7). Proportions of coniferous forest (CON) and ruderal areas (MIN) in a 1 km buffer apparently have positive effects on the population dynamic of adult Common toads, whereas effects by proportions of urban areas (URB) and cultivated land (CUL) are negative (cf. Fig. 4). The agrochemical variable ‘other herbicides’ (OHE) that includes GLY consumption is only ranked 32 of 44 variables and does not explain variance. The first agrochemical variable ranked is the herbicide ingredient group ‘amides’ (AMI), i.e. at rank 19 that also practically does not explain variance (0.1%).

With regard to juvenile life-stages, modelling resulted in only four most important predictors for the Common toad (*Bufo bufo*). The four predictors share the same ranking and exhibited the same effects (positive for CON, neutral for ACR and CUL, negative for URB) when predictors were considered with three years as well as four years delay (Table 7, Fig. 5). Also, all four of them are top ranked and showed similar effects on adults. Glyphosate use (OHE) does not explain variance and is only ranked 23 (three years) and 27 (four years), respectively. In both cases, the first listed agrochemical variable is the herbicide ingredient group of sulfonyl ureas (SUL; rank 19) explaining 0.1% variance only.

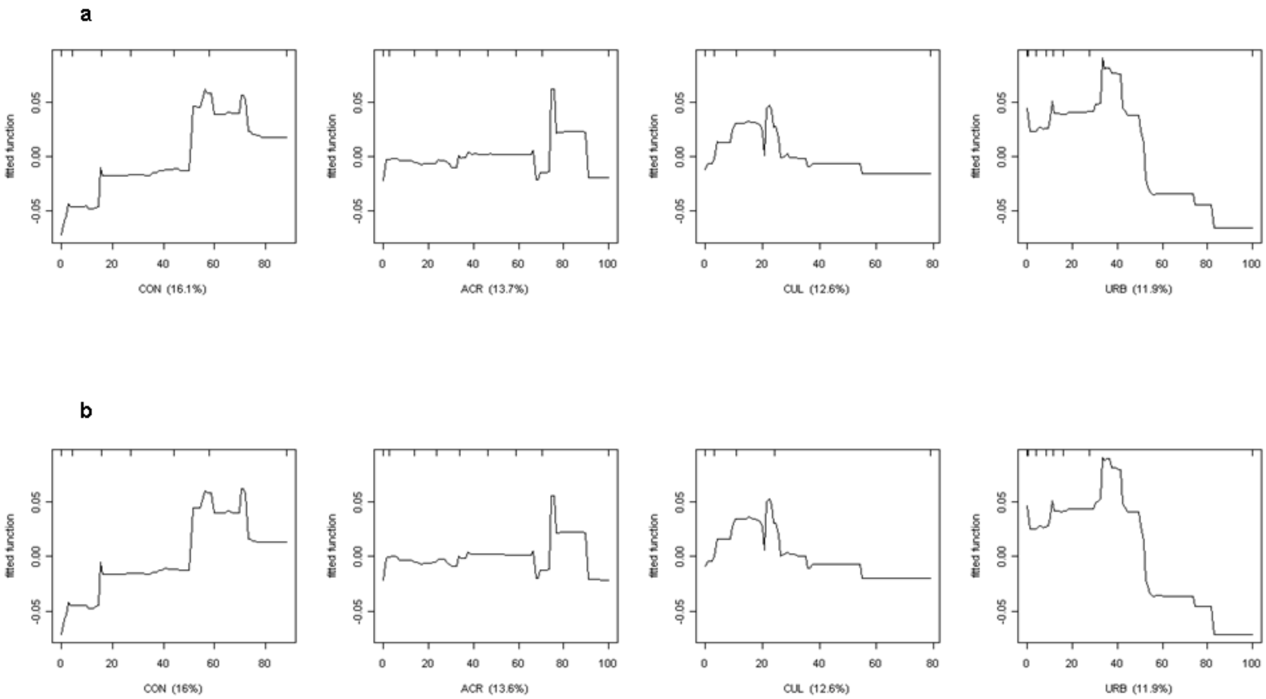

Fig. 5: Fitted functions of the most important predictors affecting juvenile life-stages of Common toads (*Bufo bufo*).
Predictor set considered three years (a) and four years prior (b), respectively. Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

For adult Common frogs (*Rana temporaria*), a large part of observed population fluctuations is explained by annual variations of all variables. This is also true when predictors were considered three years prior (Table 7). With regard to land use patterns, adults seem to be mainly affected by the presence of forests (FOR, negative) and agricultural land (ACR, positive), while for juvenile life-stages there is an additional negative effect by urban areas (URB) and vineyards (VIN) (Fig. 6).

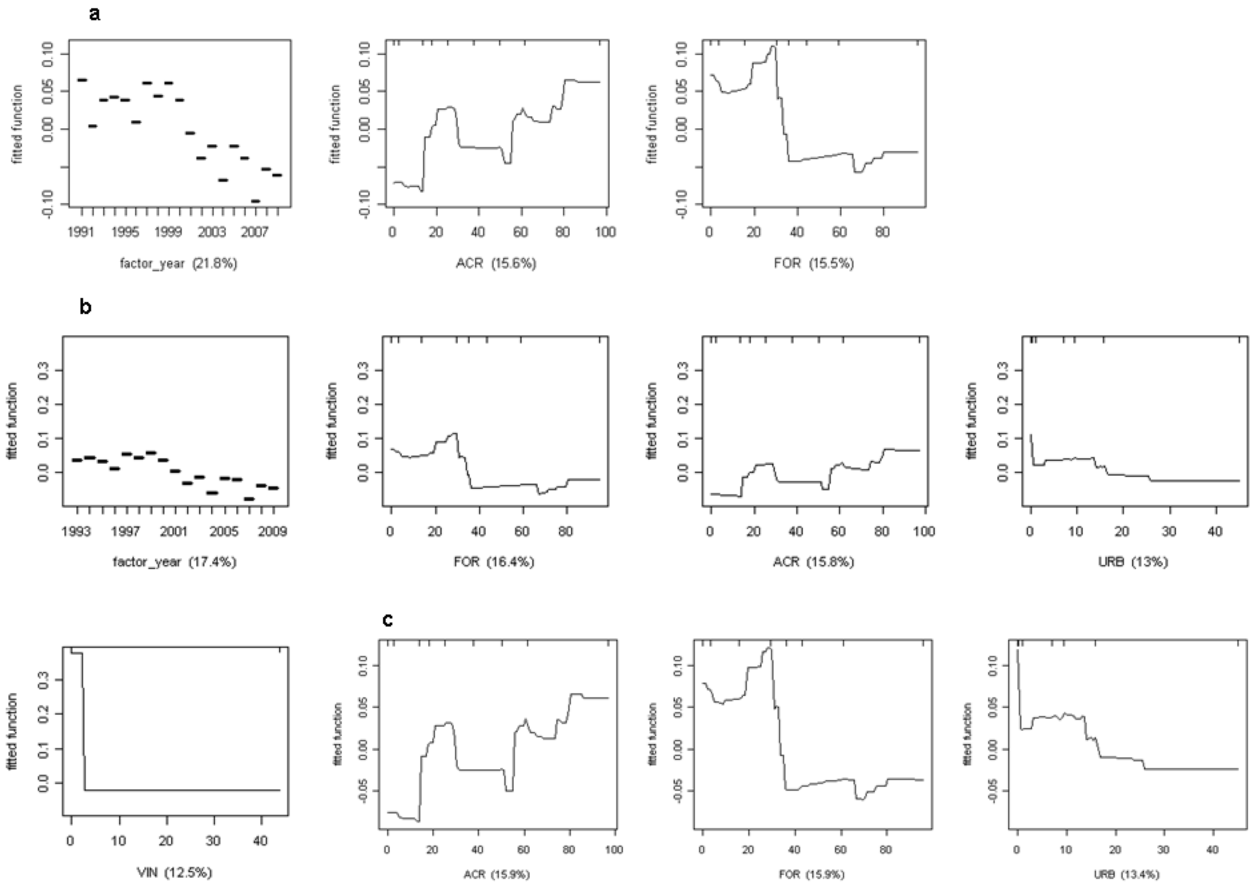

Fig. 6: Fitted functions of the most important predictors affecting adult and juvenile life-stages of the Common frog (*Rana temporaria*).
Predictor set considered one year (a), three years (b) and four years prior (c), respectively. Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

As with the juvenile life-stages of the Common toad (*Bufo bufo*), SUL is the first agrochemical variable for adult Common frogs (*Rana temporaria*) (rank 9, 0.6%). First agrochemical variables for juvenile life-stages of the Common frog (3 years prior) are ‘chlorinated hydrocarbons’ (CHL) (rank 10, 1.3%) and SUL (rank 11, 1.3%). For juvenile life-stages of the Common frogs (4 years prior), SUL is ranked 7 and explained 6.3% of variance. However, SUL is not ranked among the most important variables after simplifying the final model. Again, OHE (including GLY consumption) does not explain any variance for adults (ranked 32) or juvenile life-stages of the Common frog (rank 25 and 22 for three and four years prior, respectively; both 0.1%).

The population dynamic of adult as well as juvenile life-stages of the Moor frog (*Rana arvalis*) is largely affected by three predictors, namely the ‘factorial year’, the proportion of agricultural land with significant areas of natural vegetation (ACN) and agricultural land (ACR). Together, these three variables account for over 50% of variance in all three cases (Table 7) and their influence on the life-stages looks very similar (Fig. 7). ACN apparently has a positive effect while the other two have no clear effect. For adult Moor frogs, the first ranked agrochemical variable are the herbicidal ‘urea derivates’ (URE; rank 11, explaining 0.4% variance) and for juvenile life-stages first ranked agrochemicals are ‘inorganic fungicides and bactericides’ (INO;

rank 9, 1.5% for three years prior) and ‘sulfonyl ureas’ (SUL; rank 9, 2.1% for four years prior). In all cases, OHE explains practically no variance (rank 17 or 28, 0.1%).

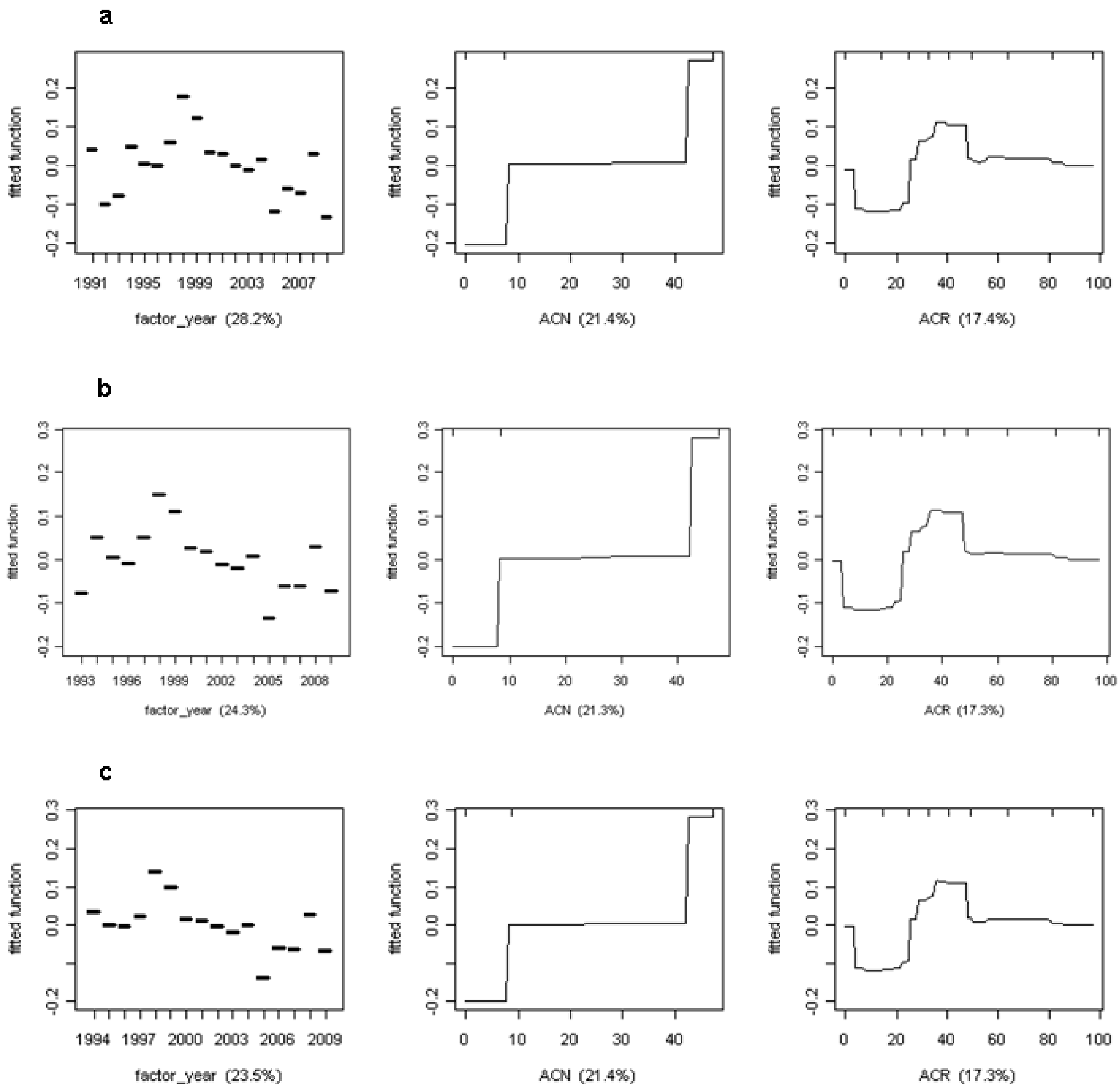

Fig. 7: Fitted functions of the most important predictors affecting adult and juvenile life-stages of the Moor frog (*Rana arvalis*).

Predictor set considered one (a), three (b) and four (c) years prior, respectively. Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

Interim conclusion

It is mentionable that none of the agrochemical predictors (not even total herbicide, insecticide, fungicide or fertiliser use) seems to affect dynamics in any of the considered amphibian populations from Germany. The herbicidal group that includes GLY consumption never explain a considerable part of variance and, therefore, apparently has no effect on the considered amphibian populations. The most important predictors are entirely presented by land use variables in a 1 km buffer around habitats, i.e. the terrestrial habitats, which seem to have considerable more impact on population dynamics. Furthermore, in many of the calculated cases the variable 'factorial year' explains a high proportion of variance, i.e. none of the considered predictors influence the population dynamic. In such cases, either other, non-considered predictors or stochastic effects are more important.

5.1.2 Amphibian populations from the USA

Amphibian populations from the USA (1990-1998)

Other than with amphibian populations from Germany, the fit of all final models in the summarised US American amphibian populations from 1991-1998 is very low (training data correlation 0.15; cross validated correlation 0.10 ± 0.02). Therefore, the value of the obtained results has to be considered as very limited or even meaningless.

Annual variations of all predictors explain almost 90% of variance for adult amphibians, i.e. no single predictor of the considered set seems to influence population dynamics. All other variables were dropped by simplification except for the total use of fungicides and bactericides (FUN). However, for this predictor no clear trend can be observed (Table 8). It is mentionable that land use change (from conventional to GM crops) apparently did not affect the considered populations and the GLY consumption as a single factor is only ranked 8 and practically does not explain variance. For juvenile life-stages (three years prior), results are principally the same as for adults (cf. Table 8), but GLY use explains no variance at all, and the total use of fungicides and bactericides apparently has a negative effect (Fig. 8). The earlier the predictor set is considered, additional data are more limited. Therefore, SPECIES (four years prior) could not be calculated due to too small sample size.

Tab. 8: Most important predictors for amphibian populations from the USA (1990-1998).

Response variable	Consumption of agrochemicals	Explanatory variable (% of explained variance)	Supposed effect
SPECIES	One year prior	factor_year (87.8)	-
		FUN_TOT (5.6)	-
	Three years prior	factor_year (71.8)	-
		FUN_TOT (19.9)	Negative

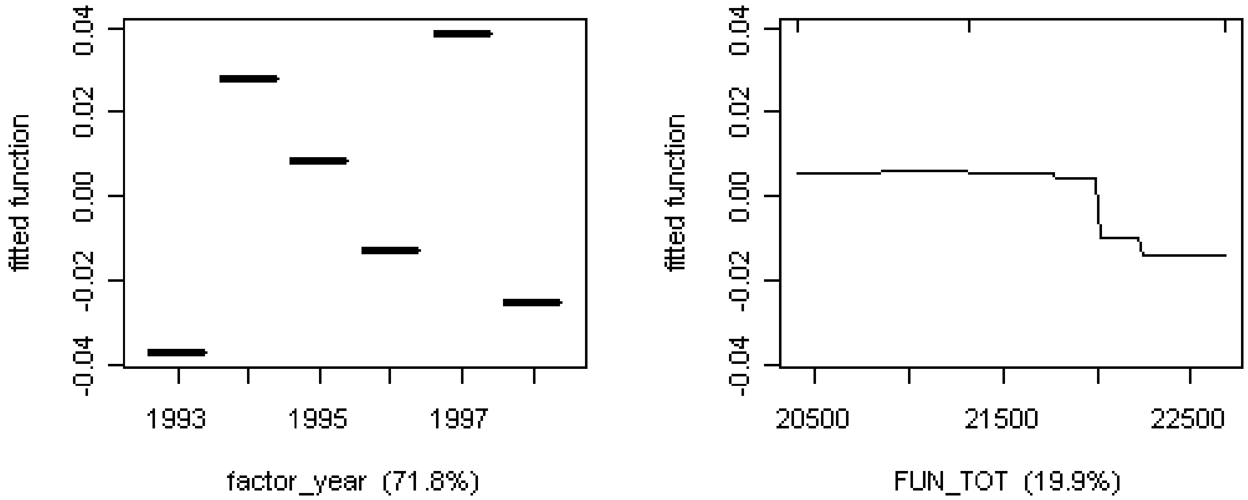

Fig. 8: Fitted functions of the most important predictors for juvenile life-stages of North American amphibian populations when the predictor set is considered three years prior.
Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

Amphibian populations from the USA (2001-2010)

With regard to the frog call survey data employed, all final models for Northern cricket frogs (*Acris crepitans*) and American toads (*Anaxyrus americanus*) show a very low fit, why results have to be considered little informative or even meaningless. Final models for Northern leopard frog (*Lithobates pipiens*) likewise do not exhibit a really convincing fit (Table 9).

Tab. 9: Final models for call surveys from the USA.

Model	Mean residual deviance	Cross validated residual deviance (± SE)	Training data correlation	Cross validated correlation (± SE)
Acris crepitans (1 year prior)	0.59	0.60 ± 0.01	0.15	0.11 ± 0.02
Acris crepitans (3 years prior)	0.59	0.60 ± 0.02	0.15	0.11 ± 0.01
Acris crepitans (4 years prior)	0.59	0.60 ± 0.02	0.15	0.11 ± 0.04
Anaxyrus americanus (1 year prior)	0.50	0.50 ± 0.01	0.13	0.10 ± 0.03
Anaxyrus americanus (3 years prior)	0.50	0.50 ± 0.01	0.13	0.11 ± 0.03
Anaxyrus americanus (4 years prior)	0.50	0.50 ± 0.02	0.13	0.10 ± 0.02
Lithobates pipiens (1 year prior)	0.03	0.28 ± 0.04	0.45	0.34 ± 0.08
Lithobates pipiens (3 years prior)	0.26	0.28 ± 0.03	0.45	0.27 ± 0.12
Lithobates pipiens (4 years prior)	0.26	0.29 ± 0.04	0.45	0.37 ± 0.08

With the Northern cricket frog again, most important predictors are the ‘factorial year’ and the fertiliser consumption that always explain about 80% of variance, no matter in which year the predictor set is considered. The amount of fertiliser consumption apparently has a slightly negative effect on juvenile-life-stages (Table 10, Fig. 9). GLY consumption (GLY) was always dropped by simplification of the final models and practically does not explain any variance (adults: rank 7 of 17, 0.5%; juvenile life-stages, three years prior: rank 5, 1%; four years prior: rank 7, 1.9%).

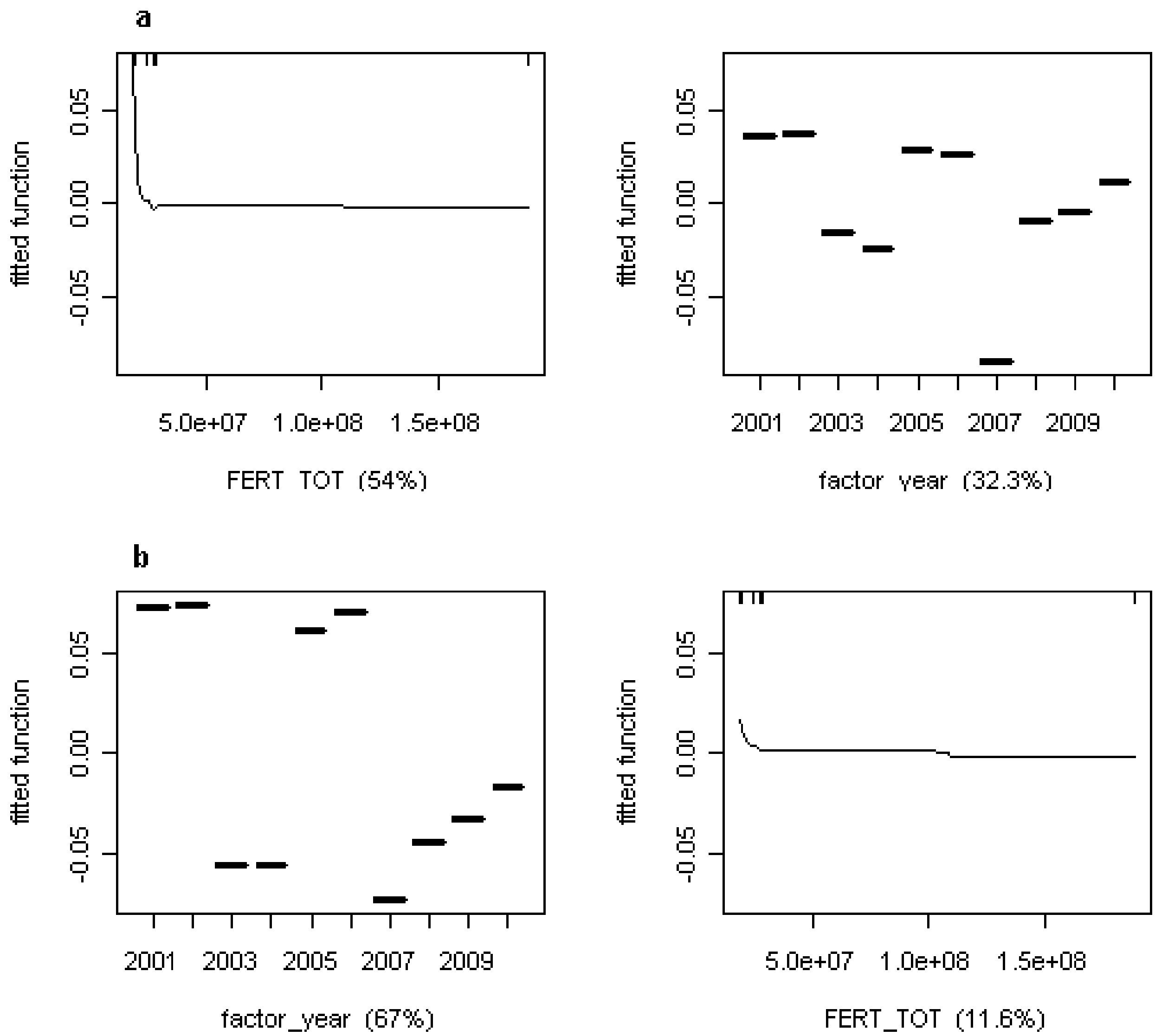

Fig. 9: Fitted functions of the most important predictors for juvenile life-stages of Northern cricket frogs (*Acris crepitans*) when the predictor set is considered three (a) and four years prior (b), respectively.
Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

Results for adult and juvenile life-stages (three years prior) of American toads are similar to the previous results, i.e. the ‘factorial year’ and the fertiliser consumption are the most important predictors (Table 10). However, when the predictor set is considered four years prior, the total applications of herbicides show a negative impact (Table 10; Fig. 10). In all cases, GLY is ranked higher than for Northern cricket frogs and explains more variance. Nevertheless, this predictor was always dropped by simplification (adults: rank 3, 2.2%; juvenile life-stages, three years prior: rank 6, 1.3%; four years prior: rank 7, 2.4%).

Tab. 10: Most important predictors after simplifying models from call surveys from the USA.

Response variable	Consumption of agrochemicals	Explanatory variable (% of explained variance)	Supposed effect
Acris crepitans	One year prior	factor_year (75.9)	-
		FERT_TOT (11.9)	-
	Three years prior	FERT_TOT (54)	Negative
		factor_year (32.3)	-
	Four years prior	factor_year (67.0)	-
		FERT_TOT (11.6)	Negative
Anaxyrus americanus	One year prior	factor_year (84.7)	-
		FERT_TOT (8.4)	Negative
	Three years prior	factor_year (68.8)	-
		FERT_TOT (18.1)	-
	Four years prior	factor_year (41.3)	-
		HER_TOT (30.2)	Negative
Lithobates pipiens	One year prior	factor_year (64.9)	-
		MES (12.8)	Negative
		GLY (11.4)	Negative
	Three years prior	factor_year (62.4)	-
		HER_TOT (17)	Negative
	Four years prior	factor_year (63.8)	-
		FUN_TOT (23.9)	-

Fig. 10: Fitted functions of the most important predictors for juvenile life-stages of the American toad (*Anaxyrus americanus*) when the predictor set is considered four years prior.
Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

In the Northern leopard frog (*Lithobates pipiens*), fit of final models at least is moderate, but the information value is still limited (Table 9). Although the ‘factorial year’ always explains the largest part of variance, adults of Northern leopard frog seem to be slightly negatively affected by application rates of metolachlor-S and GLY (Table 10, Fig. 11). Results for juvenile life-stages differ. When the predictor set is considered three years prior, the total amount of herbicide applications plays a role (Table 10; Fig. 11). GLY consumption alone is only ranked 4, explaining 3.4% variance, and was dropped by simplification of the final model. When predictors are considered four years prior, the total amount of fungicide and bactericide applications explains more than 20% of variance (Table 10), but without an apparent effect. Then, GLY is only ranked 9 (0.98%).

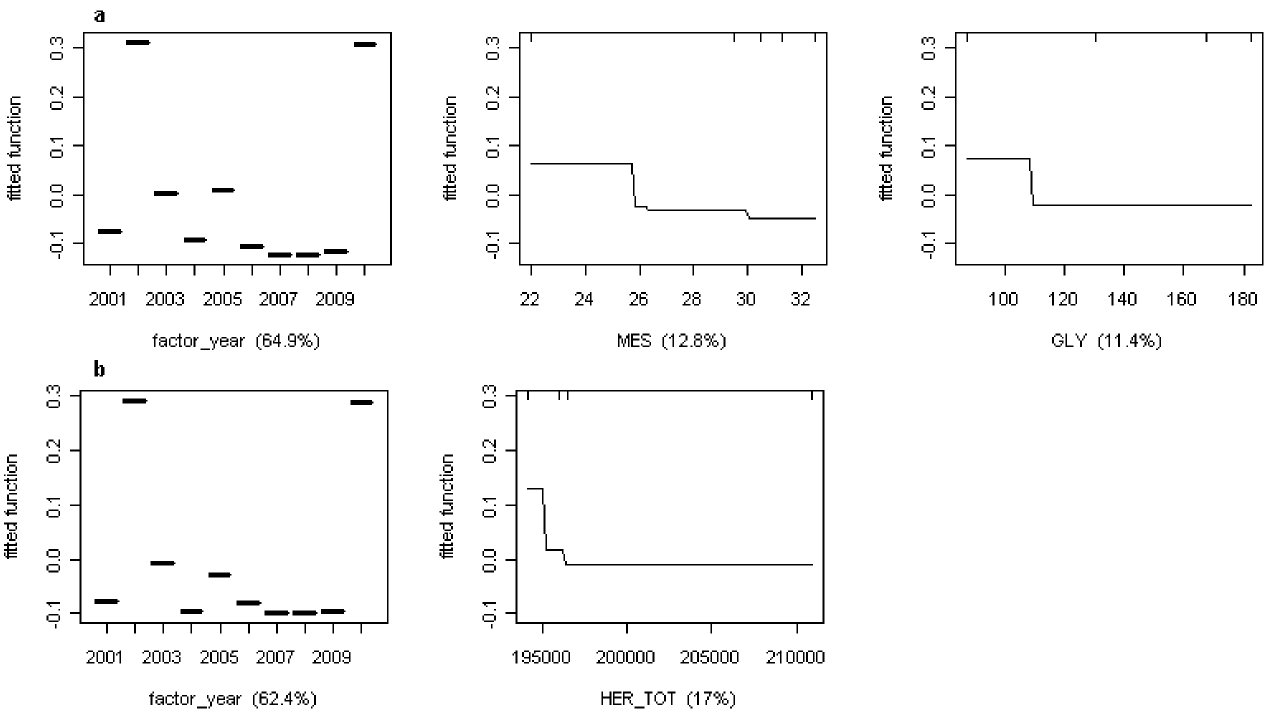

Fig. 11: Fitted functions of the most important predictors for adult (a) and juvenile life-stages (predictor set considered three years prior; b) of the Northern leopard frog (*Lithobates pipiens*).
Note that positive fitted function values suggest that species respond favorably and low values suggest the opposite.

Interim conclusion

In practically all cases, none of the considered predictors explain a significant part of variance. Hence, either other, non-considered predictors or stochastic effects are more important for population dynamics in the considered US American amphibian populations. Furthermore, the results for all amphibian populations before 2001 as well as the results from call surveys (2001-2010) for Northern cricket frogs (*Acris crepitans*) and American toads (*Anaxyrus americanus*) have to be considered as little informative to even meaningless due to very low model fits. Adults of the Northern leopard frog (*Lithobates pipiens*) are the only considered species and life-stage in the entire macroecological approach that apparently are affected by the total use of GLY. However, low to moderate model fit limits this finding.

5.2 Concentrations of glyphosate in the environment

First at all, it must be said that GLY concentrations in general only represent the risk of GBH to amphibians because we know nothing about concentrations of the added substances in the environment. Not the active ingredient (i.e. GLY salt or acid) but the added substances, especially surfactants, are mainly responsible for adverse effects on amphibians (see chapter 5.4).

Different methods are available and applied to determine GLY and AMPA in soil and water (e.g. GAUCH et al. 1989; ALFERNESS & IWATA 1994; KATOAKA et al. 1996; VREEKEN et al. 1998; CLEGG et al. 1999; BÖRJESSON & TORSTENSSON 2000; PATSIAS et al. 2001; BYER et al. 2008).[16] The most commonly used methods to determine these substances are high-performance liquid chromatography (HPLC; WINFIELD et al. 1990), precolumn derivatization followed by conventional chromatography (STRUGER et al. 2008) and liquid chromatography/tandem mass spectrometry (LC/MS/MS; LEE et al. 2002). However, all those methods are cost-intensive compared to the analysis of many other pesticides by single gas chromatography/mass spectrometry (GC/MS). Therefore, GLY is often absent from large scale pesticide monitoring (WILLIAMS & SEMLITSCH 2010). RELYEA (2006) already stated the unfortunate fact that there is very little information on how much GLY really appears in amphibian habitats.

Table 11 provides an overview of maximum concentrations of GLY found in selected environmental samples. Results are from large scale pesticide monitoring programs (TRÉGOUËT 2006, 2007; SCRIBNER et al. 2007) and scientific random surveys (PERUZZO et al. 2008; STRUGER et al. 2008; BATTAGLIN et al. 2009). Table 12 shows maximum concentrations found in experimental field studies in which the environmental fate of GBH was tested. In the field experiments, samples were mainly taken directly after application or after first downpour. Hence, these maximum concentrations are considerably higher compared to those directly found in the environment. This is because when analyses of environmental samples were conducted, the current degradation state of initial GLY applications was unknown. In field experiments, GBH were applied at normal-use scenarios. However, all studies on surface waters simulated aerial applications (FENG et al. 1990; NEWTON et al. 1984; NEWTON et al. 1994; GIESY et al. 2000; THOMPSON et al. 2004; TRUMBO 2005) whereas in the other experiments, GBH were applied on fields, except for WOOD (2001) who sprayed roads. Hence, all measurements in the environment, but only the results of five experiments (EDWARDS et al. 1980; HENKELMANN 2001; ARAÚJO et al. 2003; KJAER et al. 2003, 2009) can be directly assigned to agricultural practices. This means that the remaining results from field experiments (shown in italics in Table 12) do not allow any conclusion regarding agricultural practices.

Maximum concentrations are of particular importance because (i) GLY usually quickly dissipates in the environment and (more important) (ii) toxicity of GBH to amphibians mainly occurs within the first 24 hours (RELYEA 2005b). Concentrations in ground water, rainfall, soil and sediment samples should only represent background levels of contamination. A direct link to amphibians cannot be established because specific studies on, for instance, potential impacts of contaminated soil and sediment on amphibians are lacking (see chapter 5.4). However, neither an exposure risk to terrestrial life-stages via contact with contaminated soil nor an exposure risk to anuran larvae via ingestion of contaminated sediment can be ruled out (see chapter 6). In most cases, a contamination risk of amphibian habitats due to concentrations in the environments ground water, rainfall, soil and sediment cannot be named *a priori* because the environmental fate of GLY largely differs with soil or sediment type and local conditions. Nevertheless, a contamination is possible, for instance, due to remobilisation of GLY from contaminated soil by phosphate fertilisers (see BOTT et al. 2011) or contamination of ground water fed ponds.

Hence, from all listed maximum concentrations, particularly relevant to amphibians (and especially their larvae) are only the observed concentrations in environments that can directly be related to amphibian habitats, i.e. concentrations in (i) surface waters and (ii) run-off that could directly form ephemeral ponds (and contaminate habitats, but to an unknown degree).

It is mentionable that amphibians mainly use small water bodies for reproduction (e.g. RELYEA 2006). With regard to surface waters, the pesticide monitoring programs concentrated on streams and large standing water bodies, where it is unusual to find amphibians, whereas the scientific surveys also included ponds and wetlands. STRUGER et al. (2008) and BATTAGLIN et al. (2009) especially noted that they include amphibian ponds in their surveys. Hence, most results from pesticide monitoring programs cannot directly be related to amphibian habitats. Amounts in streams are naturally much lower than in standing waters. Likewise, one can postulate that maximum concentrations found in large water bodies of some regions have to be considerable smaller than in local small water bodies. Therefore, the highest amounts found in pesticide monitoring programs can be considered as minimum contamination level of local small water bodies.

The highest concentration measured in run-off experiments was 1,000 times higher than the smallest concentration. This seems to be related to different study designs (i.e. application rates but also soil types). The highest concentration found in surface waters was 700 µg a.e./L in environmental samples and 3,100 µg a.e./L in field experiments, but the latter concentration originate from a direct over-spraying of a water body and, therefore, cannot be directly assigned to an agricultural application. It is mentionable that the BVL (2010c) estimate a maximum concentration of 1,200 µg a.i./L = 900 µg a.e./L[17] in a small water body via drift if a GBH (360 g a.i./L) would be applicated directly beside a surface water body at the highest authorised application rate in Germany (3,600 g a.i./ha). A distance of one meter should reduce this concentration to 33.24 µg a.i./L = 24.93 µg a.e./L, a distance of 5 meters to 6.84 µg a.i./L = 5.13 µg/L. However, the size of the hypothetical water body in this estimation is unknown. For example,

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[16] Methods also exist to determine these substances in plant and animal matrices.
[17] To convert a.i. to a.e. concerning glyphosate: 1 mg a.i./L = 0.75 mg a.e./L (see GIESY et al. 2000 or

MANN & BIDWELL (1999) considered a worst-case scenario of a direct over-spraying of a very shallow water body (5 cm in depth) with a GBH (360 g a.e./L) at the highest authorised application rate in Australia (3,816 g a.e./ha, i.e. similar to Germany) that would result in 7,600 µg a.e./L (21.1 mg/L of the whole product). Such shallow water bodies like flooded fields are commonly used for reproduction by Australian amphibian species (MANN & BIDWELL 1999), but this is also true for some (strictly protected) species, which occur in Germany (e.g. European treefrogs, FLOTTMANN & LAUFER 2004).

Overall, published estimated worst-case scenarios in surface waters range from 0.9 to 7.6 mg a.e./L: 0.9 mg a.e./L (BVL 2010c), 1.4 and 2.7 mg a.e./L (SOLOMON & THOMPSON 2003), 2.8 mg a.e./L (GIESY et al. 2000), 2.9 mg a.e./L (PERKINS et al. 2000) and 7.6 mg a.e./L (MANN & BIDWELL 1999).When compared to the highest concentrations found in the environment (0.7 mg a.e./L) and field studies (3.1 a.e.mg/L), these estimates seem to represent worst-case scenarios satisfactorily.

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http://www.pitt.edu/~relyea/Site/Roundup.html).

Tab. 11: Maximum concentrations of GLY directly found in the environment.

Maximum concentration in different environments	Region	Reference
Ground water [µg a.e./L]		
24.0	Europe (France)	TRÉGOUËT 2007
4.7	North America (USA)	SCRIBNER et al. 2007
6.8	Europe (France)	TRÉGOUËT 2006
Surface water [µg a.e./L]		
700	South America (Argentina)	PERUZZO et al. 2008
427	North America (USA)	SCRIBNER et al. 2007
328	North America (USA)	BATTAGLIN et al. 2009
165	Europe (France)	TRÉGOUËT 2006
40	North America (USA)	STRUGER et al. 2008
17	Europe (France)	TRÉGOUËT 2007
Rainfall [µg a.e./L]		
1.1	North America (USA)	SCRIBNER et al. 2007
Soil [µg a.e./kg]		
4,000	South America (Argentina)	PERUZZO et al. 2008
427	North America (USA)	SCRIBNER et al. 2007
Sediment [µg a.e./kg]		
5,000	South America (Argentina)	PERUZZO et al. 2008

Tab. 12: Maximum concentrations of GLY found in experimental field studies.
Note that results from field experiments that cannot be assigned to agricultural practices are written in italics.

Maximum concentration in different environments	Region	Reference
Surface water [µg a.e./L]		
3,100	North America (USA)	TRUMBO 2005
1,950	North America (Canada)	THOMPSON et al. 2004
1,700	North America (Canada)	GIESY et al. 2000
1,237	North America (USA)	NEWTON et al. 1994
270	North America (USA)	NEWTON et al. 1984
162	North America (Canada)	FENG et al. 1990
Run-off [µg a.e./L]		
5,200	North America (USA)	EDWARDS et al. 1980
736	North America (USA)	WOOD 2001
49.5	Europe (Germany)	HENKELMANN 2001
31	Europe (Denmark)	KJAER et al. 2009
5.1	Europe (Denmark)	KJAER et al. 2003
Soil [µg a.e./kg]		
4,670	North America (USA)	NEWTON et al. 1994
2,376	South America (Brazil)	ARAÚJO et al. 2003
Litter-covered soil [µg a.e./kg]		
1,400	North America (USA)	NEWTON et al. 1994

North America

In the USA, agrarian GLY application increased from 6-8 millions of pounds of active ingredient in 1987 to 180-185 in 2007 (ASPELIN 1997; GRUBE et al. 2011). This immense increase is mainly caused by the replacement of conventional crops with GLY-resistant crops in soybean, cotton and maize production (e.g. DUKE & POWLES 2008; BATTAGLIN et al. 2009) and has now reached nearly 100% for soybean. Although GLY is also used with conventional crops and for other purposes, the increase in GLY use is highly correlated with cultivation of HR crops (cf. chapter 3). This implies that contamination of the environment with GLY is less likely in countries of no GM crop cultivation.

SCRIBNER et al. (2007) conducted a large pesticide monitoring program on behalf of the US Geological Survey (USGS) to document the occurrence, fate and transport of GLY and AMPA. They analysed more than two thousand ground- and surface water samples, 14 rainfall samples and nearly two hundred soil samples from different states between 2001 and 2006.[18] Generally, GLY was detected more frequently in surface than in ground water, but maximum concentrations differed for both: (i) About 50% of all surface water samples at the Leary Weber Ditch Basin contained the highest measurement of 427 µg a.e./L. (ii) In the NAWQA program, GLY was detected in 196 out of 608 surface water samples, but only in 28 out of 485 ground water samples with a ground water maximum of 9.7 µg a.e./L and a surface water maximum of 0.33 µg a.e./L (detected in two out of 27 samples). (iii) 63 out of 171 surface water samples taken from 51 midwestern streams contained GLY with a maximum concentration of 8.7 µg a.e./L compared to 4.7 µg a.e./L for the maximum in ground water samples. The latter is similar to the Leary Weber Ditch Basin (i.e. 0.67 µg a.e./L). In this area, rainfall data were also taken. In 12 out of 14 rainfall samples, GLY was detected with a maximum of 1.1 µg a.e./L. Together, all data indicated that trace levels of GLY may persist in soil and accumulate over years.

STRUGER et al. (2008) ran a bi-weekly investigation into surface water at 56 different sites including rivers, small streams and low-flow wetlands in the years 2004 (May to December) and 2005 (April to November) across southern Ontario, Canada. Thirty sites were considered habitats of amphibians. In 23 potential amphibian sites, GLY was detected. Mean concentrations were relatively low with the maximum concentration measured being 40.0 µg a.e./L.

BATTAGLIN et al. (2009) investigated the occurrence of GLY, AMPA and additional pesticides in vernal pools and adjacent flowing water for the period of 2005-2006 in Washington D.C., Maryland, Iowa and Wyoming. Vernal pools are ephemeral wetlands that dry up during warmer and drier times of the year. Although these kind of water bodies are considered 'critical habitats' for many North America amphibian species (BATAGLLIN et al. 2009), they are not always protected by pesticide label requirements for no-spray buffer zones (MANN et al. 2003; THOMPSON et al. 2004). The highest pesticide concentration measured in the study by BATTAGLIN et al. (2009) was of GLY (328 µg a.e./L; see Table 11). However, BATTAGLIN et al. (2009) especially mentioned that this maximum was measured in a vernal pool that was directly over-sprayed with the GBH Accord® for non-indigenous plant control. Because this formulation is designed and labelled for application adjacent to water bodies usually not used in agriculture, this maximum concentration is not directly comparable with the entry of GLY from agrarian use. The next highest amount was only 12 µg a.e./L.

[18] The samples belonged to different monitoring programs, e.g. the ‘National Stream Quality Accounting Network’ (NASQAN) program, the ‘National Water Quality Assessment’ (NAWQA) program and a project by BAKER et al. (2006) at the Leary Weber Ditch Basin, i.e. part of the White River Basin (Indiana) with adjacent agriculture.

There are additional data for North America from experimental field studies. EDWARDS et al. (1980) investigated the concentrations of GLY in run-off by applying Roundup® (probably Roundup Original®) to watersheds at rates of 1.10 to 8.96 kg/ha. These rates should simulate no-tillage farming of fescue and maize. However, it is unclear if EDWARDS et al. (1980) meant kg a.e./ha or kg formulation/ha. One day after application at the highest rate, the highest concentration (5,200 µg a.e./L) was measured in natural rainfall samples. GLY residues could be detected for up to four months.

NEWTON et al. (1984) evaluated GLY residues in a forest brush field ecosystems 55 days after an aerial application of 3.3 kg a.e./ha. The highest concentration found in a stream directly after application was 270 µg a.e./L, but this concentration decreased rapidly. Overall, concentrations were higher in sediments than in water and persisted longer.

FENG et al. (1990) treated 45 ha of coastal Canadian watershed with 2.0 kg Roundup® (probably Roundup Original®)/ha per aerial application. Maximum GLY concentrations were 162 µg a.e./L for stream water and 6.80 µg a.e./g sediment. They occurred in two intentionally over-sprayed tributaries. Concentrations dissipated to < 1 µg a.e./L within 96 hours after application. Buffered streams only exhibited GLY concentrations ranging from 2.4-3.2 µg a.e./L (see also THOMPSON et al. 2004).

NEWTON et al. (1994) treated forest sites with 4.12 kg a.e./ha per aerial application. Residues were highest in upper crown foliage as the overstory reduced exposure of understory vegetation and streams. Most residues in streams were close to the detection limit or undetectable in 3-14 days. However, maximum concentration from a site when the water was fully exposed was 1,237 µg a.e./L. Residues in soils were highest where cover was sparse and where litter was removed. No residues were detectable in soil 409 days after treatment; movement below 15 cm was negligible. AMPA appeared at low levels in all degrading matrices, including sediments, soon after GLY was deposited. In ponds, both GLY and AMPA remained bound to sediments.

PAVEGLIO et al. (1996) treated 1 ha-plots with 4.7 L Rodeo® per ha with aerial application and additionally with 0.9 L pure surfactant per ha, i.e. X-77®, to combat the non-native smooth cordgrass (*Spartina alterniflora*) at three estuary sites in Washington State (USA). They measured concentrations of GLY, AMPA and X-77®, i.e. nonylphenol polyethoxylates. Nonylphenol polyethoxylates were not detected in seawater samples, but they were detected in sediment samples where 42% of the measured concentrations declined one day after treatment. GLY was detected in seawater and sediment samples. 73% of GLY in seawater declined one day after treatment. However, these 'declines' were in consequence of the first high tide after application. In sediment samples, GLY concentrations declined by 51-72% 119 days after treatment. However, they named only mean values of GLY concentrations in sediment and seawater, i.e. the maximum mean values of 2.82 µg a.e./g in marine sediment and 25.6 µg a.e./L in seawater. Hence, these values are not listed in Table 12.

WOOD (2001) conducted an assessment on the run-off in the management of roadside vegetation in Oregon (USA). The applied herbicides included Roundup® (probably Roundup Original®). Application rates were similar to those the Oregon Department of Transportation uses for vegetation management. A heavy rainfall was first simulated one day after application. Run-off for GLY was only 0.9-2.1%. However, concentrations measured directly after simulating the rainfall range from 323 to 736 µg a.e./L. Two weeks after application, the procedure was repeated and concentrations ranged from 16 to 42 µg a.e./L. In a second experiment, they repeatedly applied the herbicides on the road and concentrations were measured in the hard shoulder, the drainage ditch and the nearby stream one to four months after application and after natural rainfalls. GLY was never detected, whereas the other two herbicides (diuron and bromacil) could be detected at low concentrations, even three months after application.

For the experimental design of the study by THOMPSON et al. (2004), see chapter 5.4.3. TRUMBO (2005) applied Rodeo®, which is also designed and labelled for application adjacent to water bodies, to a small pond using a vehicle-mounted hose-gun sprayer and a typical tank-mix concentration of 1% herbicide. The herbicide use rate was five pints/surface acre. One hour after application, mean GLY concentration in the pond was 1.83 mg a.e./L, with a maximum concentration of 3.1 mg a.e./L.

South America

There is very limited information on environmental GLY concentrations in South America. For example, PÉREZ et al. (2007) found no information of measured GLY concentrations in standing waters in Argentina. However, the highest concentrations of GLY in the environment were measured in this country. PERUZZO et al. (2008) took water, soil and sediment samples from a GM soybean cultivation area. This site was in an area of high agricultural production. Here, soils are well drained and rich in organic matter, and sediments have a higher content of clay than the soil. Downstream, the water column loses a significant proportion of these colloids and remaining clay particles have a great capacity to retain toxicity. Samples were collected in a first-order stream that flows through the cultivation area and four surface streams. GLY concentrations in water samples ranged from 0.1 to 0.7 mg a.e./L. A significant increase in GLY concentrations was shown in relation to rainfall. Higher amounts were also observed directly after herbicide application before sowing (no-tillage farming). The highest concentration in soil samples was > 4,000 µg a.e./kg and in sediment samples about 5,000 µg a.e./kg. PERUZZO et al. (2008) also modelled values of GLY after rain events for the different sites using the ‘SoilFug’ multimedia model of the Canadian Environmental Modelling Centre. The results showed a high concordance with those observed in the field.

In addition, it is mentionable that KAISER (2011) investigated the pesticide drift from nearby agriculture into the protected areas of the Maya Mountains in Belize, Central America, by collecting water samples from phytotelmata, i.e. water bodies held by plants such as bromeliad tanks. She detected organophosphates/carbamates and GLY at all sites. The highest mean concentration at a sample site was 1.7 µg a.e./L. Phytotelmata are the breeding habitat of several amphibian species. For example, in Belize the endangered Bromeliad frog (*Bromeliohyla bromeliacia*) use bromeliad tanks (CRUZ et al. 2008). Similar concentrations by pesticide drift could also be assumed for some phytotelmata in South America. Beside several endangered dendrobatid frogs, the whole *Scinax perpusillus* species group exclusively reproduces in bromeliad tanks (PEIXOTO 1987).

In an experimental field study, ARAÚJO et al. (2003) found different concentrations of GLY in two types of Brazilian soils with different histories of GLY application (0, 6 or 11 years). They sampled the soils up to a depth of 10 cm and applied 2.16 mg a.e./kg on 75 g sub-samples which were then incubated in the dark for about one month at 25°C. Highest amounts (2.38 mg a.e./kg) directly after application of GLY were detected in samples of a sandy clay soil with reported historical GLY application of six years and smallest amounts (1.45 mg a.e./kg) in a clay soil with reported historical GLY application of 11 years. In contrast, after 32 days of biodegradation, GLY concentrations of only 0.14 mg a.e./kg could be detected in the sandy clay soil (the smallest amount of all), but 0.46 mg a.e./kg was detected in the clay soil (the highest amount of all).

Europe

15% (76 out of 510) of surface water samples taken in France between 2003 and 2004 contained GLY with concentrations ranging from 2.0 µg a.e./L to 165 µg a.e./L (median 3.6 µg a.e./L) (TRÉGOUËT 2006). In a follow-up study the year after, GLY was detected in 43 out of 871 surface water samples. Maximum and minimum concentrations were 17 µg/L and 2.1 µg/L respectively (median 3.1 µg/L) (TRÉGOUËT 2007).

Between 1999 and 2002, KJAER et al. (2003) conducted five field experiments in Denmark. They applied Roundup 2000® on an acre with coarse, sandy soil, prior to the sowing of winter rye. Unfortunately, the authors did not name the exact applied amount per hectare. The leaching risk was negligible at this site as the measured concentrations did not exceed 0.1 µg a.e./L. In a subsequent study, they applied 1.44 kg a.e./ha on a field with a loamy soil using Roundup Bio® (4.0 L/ha). Five days after spraying, 12 mm of precipitation caused about 2 mm of drainage run-off with a flow-proportional (i.e. drainage run-off induced by a sudden storm event) GLY concentration of 4.7 µg a.e./L and a time-proportional (i.e. continuous drainage run-off during the whole drainage season) concentration of 1.9 µg a.e./L. The concentration constantly decreased during the leaching period, which is from October to July. The weighted average concentration of GLY was 0.13 µg a.e./L in the drainage water. KJAER et al. (2003) stated that normally concentrations of GLY in drainage water might have been higher because November and December were much drier than usual in the year the study was conducted. Here, GLY was detected in ground water samples at concentrations below 0.1 µg a.e./L. In a third study, KJAER et al. (2003) applied an unknown amount of Roundup Bio® to combat couch grass in October in a study site with a sandy, loamy soil. At the end of the month, the field was ploughed and sown with field peas. Weighted average GLY concentration in the drainage run-off was 0.54 µg a.e./L with maximum concentration 2.1 µg a.e./L. Here, leaching of GLY (maximum 0.03 µg a.e./L, mean 0.01 µg a.e./L) was also observed in the next year. The highest concentration in ground water samples was 0.01 µg a.e./L. In a fourth experiment, KJAER et al. (2003) applied an unknown amount of Roundup 2000® in August to an acre with a sandy, loamy soil. At the end the month, winter wheat was sown. The following year in October, the field was sprayed one more time with an unknown amount of Roundup 2000® and ploughed 12 days later. In the following May, sugar beet was sown. In the end of October of the same year, the field was ploughed one more time and spring barley was sown in the following May. GLY concentrations in drainage run-off (also in the first) and ground water samples did not exceed 0.1 µg a.e./L. In a fifth experiment, 4.0 L/ha Roundup Bio® was applied in September to a field with pea residues on sandy clay soil. In October, the residues were removed, the field was ploughed and winter wheat was sown. Mean GLY concentration in run-off samples was only 0.04 µg a.e./L, despite the highest concentration (5.1 µg a.e./L) measured after 34 mm of precipitation. In this experiment, GLY was not detected in ground water samples. As already mentioned, the type of soil is an important factor for the adsorption and biodegradation of GLY. Here, KJAER et al. (2003) explained low leaching risk by a matrix rich in aluminium and iron, providing good conditions for sorption and degradation. Conversely, the findings indicate that GLY can leach through the root zone at concentrations exceeding 0.1 µg a.e./L in loamy soil when applied in late autumn (leaching was also negligible on a sandy, loamy soil when applied in August). KJAER et al. (2003) stated that this was apparently attributable to a pronounced macropore flow shortly after application in combination with a limited sorption and degradation capacity. In principal the same conclusions were made for the following assessments in the years 2003-2004 (KJAER et al. 2009).

HENKELMANN (2001) conducted studies on the drainage run-off of GLY in Germany. The drainage run-off was collected at the footslope of three slightly sloping field sites. Three L/ha of an unknown GBH (360 g a.e./L) were applied in April on the catch crops, shortly before silage maize was sown. Samples of drainage water and soil were analysed from April to June of the subsequent year. In 34 out of 55 drainage water samples, GLY was detected, both directly after application and especially in autumn due to high precipitation and the maize harvest (maximum concentration 49.5 µg a.e./L). During the growth of the maize, both GLY concentrations (mean 1.84 µg a.e./L.) in run-off were negligible.

Interim conclusion

Taken together, GLY concentrations can only represent risks to amphibians because formulations are generally more dangerous to amphibians, but little is known about environmental fate and nothing about environmental concentrations of added substances. GLY is underrepresented in pesticide monitoring because determination methods are relatively cost-intensive. Hence, also little information is available on GLY contamination of amphibian habitats.

It is difficult to relate concentrations in ground water, soil, sediment or rainfall directly to amphibians. The reason is that neither an exposure risk to amphibians nor a contamination risk of amphibian habitats can be deduced from such amounts because specific studies are lacking and GLY's environmental fate strongly differs. Nevertheless, impacts on amphibians and contamination of amphibian habitats at a later stage are possible.

Especially important are therefore amounts in environments that can be related to aquatic amphibian habitats, i.e. concentrations in surface waters and run-off. Although directly in the environment mainly large water bodies were investigated (streams or lakes) that are mainly not suited as amphibian habitat, their maximum GLY concentrations should represent a minimum contamination of local, small amphibian ponds.

The maximum amounts are important because GBH quickly affect amphibians. Data on maximum GLY concentrations can be divided into three main groups: (i) amounts that were measured directly in the environment, (ii) results from field experiments that investigated the environmental fate of GLY and (iii) estimated concentrations for worst-case scenarios. Concentrations from field experiments are usually higher than those directly measured in the environment. The reason is that GLY is applied in a controlled manner in field experiments whereas the degradation state of GLY residues is unknown when samples were taken directly from the environment. Hence, it can be postulated that local contamination is temporarily considerably higher than the measured amounts. Therefore, results from field experiments are mainly better suited to assess the risk of water contamination by GLY. However, it has to be noted that most field studies cannot directly be related to agrarian use of GLY.

The highest concentration that were directly found in the environment was 0.7 mg a.e./L in an Argentinian stream after heavy rainfalls. The maximum concentration in a field experiment resulted from direct over-spraying (3.1 mg a.e./L). Highest amount in run-off from field was 5.2 mg a.e./L. The expected environmental concentrations in surface waters based on worst-case scenarios (direct over-spraying) range from 0.9 to 7.6 mg a.e./L and represent maximum concentrations in the environment and field experiments satisfactorily.

Overall, surface water contamination levels that were found in countries where GM crops are already authorised were sometimes considerably higher than those found in Europe. However, the highest ground water concentration was found in France. This seems to be related to the fact that GLY already is widely used besides agriculture (e.g. in winegrowing).

5.3 Concentrations of AMPA in the environment

First, it should be noted that although GLY is often the main source of AMPA, it is not the only source since other phosphonate compounds can also be degraded to AMPA (e.g. NOVACK 1997; SKARK et al. 1998; BOTTA et al. 2009). For example, phosphonic acids were used as detergents in large amounts in cleaning processes and can degrade to AMPA (SKARK et al. 1998). The proportion of AMPA that can be linked directly to GLY and AMPA that can be linked to other phosphonate compounds are unknown.

Table 13 overviews the maximum concentrations of AMPA found in the environment, and Table 14 shows concentrations found in experimental field studies. Results are from the same studies as for GLY concentrations. The highest concentrations important for potential direct impact on amphibians and their aquatic habitats (see chapter 5.2) are 400 µg/L in surface waters and 7.2 µg/L in run-off. For AMPA, the maximum concentration measured in surface waters directly in the environment is nearly ten times higher than those from field studies. This seems to be related to the fact that in the mainly short-term field studies the environmental fate of GLY was tested and the measured amounts of AMPA originate from degradation of the applied GLY. Hence, amounts of AMPA from field studies are also much lower than the GLY concentrations (cf. Table12).

It is noticeable that the amounts of AMPA that were directly found in the environment are generally much lower than GLY concentrations, but AMPA has been found more frequently in samples. The maximum GLY concentration in surface waters is more than 100 times higher than the maximum AMPA concentration. Therefore, one can postulate that GLY contamination of aquatic amphibian habitats due to direct over-spraying, drift or run-off is higher than AMPA contamination due to run-off or leaching. Apparently, run-off and leaching of AMPA is prevented by its stronger adsorption behaviour than GLY (RUEPPEL et al. 1977). Conversely, KJAER et al. (2003, 2009) observed long-term leaching of AMPA. Furthermore, long persistence and accumulation of AMPA has been observed (e.g. HENKELMANN 1992; MAMY et al. 2008, 2010). Hence, contamination of aquatic amphibian habitats is possible and high concentrations of AMPA in soil samples are therefore also worth mentioning.

Tab. 13: Maximum concentrations of AMPA directly found in the environment.

Maximum concentration in different environments	Region	Reference
Ground water [µg/L]		
2.6	North America (USA)	SCRIBNER et al. 2007
Surface water [µg/L]		
66.0	North America (USA)	STRUGER et al. 2008
48.1	Europe (France)	TRÉGOUËT 2006
41.0	North America (USA)	BATTAGLIN et al. 2009
18.8	Europe (France)	TRÉGOUËT 2007
8.7	North America (USA)	SCRIBNER et al. 2007
Rainfall [µg/L]		
0.5	North America (USA)	SCRIBNER et al. 2007
Soil [µg/kg]		
956	North America (USA)	SCRIBNER et al. 2007

Tab. 14: Maximum concentrations of AMPA found in experimental field studies.

Maximum concentration in different environments	Region	Reference
Surface water [µg/L]		
< 3.0	North America (USA)	NEWTON et al. 1994
0.05	North America (USA)	NEWTON et al. (1984)
Run-off [µg/L]		
7.2	Europe (Germany)	HENKELMANN 2001
5.4	Europe (Denmark)	KJAER et al. 2003
1.6	Europe (Denmark)	KJAER et al. 2009
Soil [µg/kg]		
788	South America (Brazil)	ARAÚJO et al. 2003
510	North America (USA)	NEWTON et al. 1994
Litter-covered soil [µg/kg]		
680	North America (USA)	NEWTON et al. 1994

North America

The highest concentration of AMPA in surface water samples of vernal pools measured in the study by BATTAGLIN et al. (2009) was 41 µg/L. SCRIBNER et al. (2007) detected AMPA at higher frequency than GLY. Furthermore, AMPA was verified more frequently in surface than in ground water samples. AMPA was also detected in 313 out of 608 surface water samples of the NAWQA program, with the highest concentration of 8.7 µg/L. In contrast, AMPA could only be found with the highest measurement of 0.38 µg/L in samples of the NASQAN program (17 out of 27 samples). Out of 171 samples collected in 51 midwestern streams, AMPA was confirmed in 117 samples with a maximum concentration of 3.6 µg/L. AMPA was also shown to occur in 47 out of 485 ground water samples of the NAWQA program; here with a maximum concentration of 0.62 µg/L. The highest measurement in ground water was 2.6 µg/L, taken in the Leary Weber Ditch Basin. AMPA was also present in 12 out of 14 rainfall samples from this study site. Maximum concentration of AMPA during preapplication was 23 µg/kg soil and a maximum measurement in soil samples of 956 µg/kg. Similarly to GLY, data from BAKER et al. (2006) indicate that even trace levels of its main degradation product may persist in soil over years. Furthermore, residues of AMPA could be detected in rainfall samples.

Despite the results of SCRIBNER et al. (2007) and BAKER et al. (2006), STRUGER et al.

(2008) did not observe residues of AMPA in surface water samples in any of the 30 amphibian habitats studied. However, AMPA was confirmed in samples from other study sites; the highest concentration was 66.0 µg/L.

PAVEGLIO et al. (1996) found that 42% of AMPA concentrations in seawater declined one day after treatment, i.e. the first high tide. In contrast to GLY, no differences in AMPA concentrations in sediment samples were found from spray day to 119 days after treatment, showing the high persistence of AMPA in sediments. Again, they only stated mean values of AMPA and no maximum concentrations, i.e. maximum mean values of 0.161 µg AMPA/g marine sediment and 1.32 µg AMPA/L seawater.

South America

There is very little information available. Thirty-two days after application, ARAÚJO et al. (2003) measured AMPA in soil samples with the highest amount (0.79 mg/kg) in a historically (six years previously) GLY treated sandy clay soil.

Europe

In France, AMPA had the highest frequency in surface water samples among all pesticides and pesticide degradation products. In 250 out of 510 samples, AMPA could be confirmed with a maximum and minimum concentration of 48.1 µg/L and 2.1 µg/L (median 3.0 µg/L), respectively (TRÉGOUËT 2006). In 2005, AMPA occurred in 79 out of 871 surface water samples; maximum and minimum concentrations were 18.8 µg/L and 2.1 µg/L, respectively (median 2.8 µg/L) (TRÉGOUËT 2007).

In a field study in Denmark by KJAER et al. (2003), the highest AMPA concentrations in drainage run-off were 5.4 µg/L, 0.06 µg/L (flow-proportional) and 0.14 µg/L (time-proportional). The AMPA concentration in run-off was lower than the GLY concentration, but more stable during the leaching period. The finding that leaching differs with the soil types also applies to AMPA. In the Danish study, AMPA did not exceed concentrations > 0.1 µg/L at the sites where observed GLY leaching was low. Principal findings of the studies conducted from 1999 to 2002 correspond well with those obtained in a follow-up study in 2003 and 2004 (KJAER et al. 2009).

In 23 out of 55 samples from Germany, HENKELMANN (2001) detected AMPA with a mean concentration equal to 0.33 µg/L (maximum was 7.22 µg/L). The observation that the measured amounts could be correlated with rainfall events and that leaching was negligible during the growth of maize also accounts for AMPA concentrations in run-off.

Interim conclusion

Taken together, the contamination level of AMPA cannot be directly related to GLY because other phosphonate compounds can also be degraded to AMPA. However, AMPA concentrations found in field studies should mainly originate from the prior applied GLY. Because most of the experiments were short-term and therefore GLY was not fully degraded, AMPA concentrations are higher in the environment.

Overall, amounts of AMPA that were directly found in the environment are generally much lower than GLY concentrations. Hence, GLY contamination, for instance, due to drift seems to be more important. Nevertheless, AMPA was usually found more frequently and long-term contamination by AMPA is possible.

According to CAREY et al. (2008), AMPA is not more toxic than GLY to fish and other standard test organisms. Unfortunately, it is difficult to relate the measured AMPA concentrations to amphibians because of lacking studies (see chapter 5.4).

5.4 Laboratory studies, mesocosm and field experiments

Amphibians do not belong to the standard test organisms of ecotoxicological risk assessments required for the approval of pesticides. Laboratory results from fishes and invertebrate aquatic organisms (e.g. daphnia) are being transferred to aquatic amphibian larvae; results from mammals and birds are transferred to terrestrial life-stages of amphibians (ALDRICH 2009; RELYEA 2011). The ecology, biology and life cycles of these standard test organisms differ remarkably from those of amphibians, so that an extrapolation is questionable (e.g. RELYEA 2011), but by comparing ecotoxicological endpoints, ALDRICH (2009) found that standard assessments with fish and aquatic invertebrates should widely cover the acute toxic risk of pesticides to tadpoles when regular safety factors are being considered (see also Table 15; for GLY isopropylamine salt see comments in the table legend). Chronic effects cannot be compared due to strongly different study designs (especially different considered endpoints). With regard to terrestrial life-stages, substances are orally administered to mammals and birds, but dermal uptake seems to be more important for amphibians: GLY is absorbed 26 times faster by amphibians than by mammals (QUARANTA et al. 2009).

Tab. 15: Comparison of the lowest LC50 values (mg a.e./L) of GLY and a common GBH (Roundup Original®) to larval anurans and standard aquatic test organisms.

Test substance	Anuran larvae	Aquatic invertebrates	Fishes	References
GLY acid (CAS # 1071836)	78	128	71.4	MANN & BIDWELL 1999; DILL et al. 2010
GLY isopropylamine salt (CAS # 38641940)	6.5 [a)] / >17.9 [b)] / >400 [c)]	428	>460	MANN & BIDWELL 1999; HOWE et al. 2004; TRUMBO 2005; DILL et al. 2010
Roundup Original® (MON 2139)	0.3	3.0	1.3	DILL et al. 2010; KING & WAGNER 2010

Legend: Note that tests were conducted without additional stressor and lasted with fish for 96h, with aquatic invertebrates for 48h and with amphibian larvae for only 24h. Hence, lower $LC50_{96\text{-}h}$ values for tadpoles are possible.

CAS # = Chemical Abstract Service number; MON = Monsanto

a) TRUMBO (2005) exposed tadpoles to a Rodeo®/R-11® mixture and calculated the LC50 value for GLY isopropylamine salt from these results. Hence, no direct tests with the a.i. were conducted and the value should be viewed with caution.

b) The highest concentration that was used by HOWE et al. (2004) was 18 mg a.e./L to maintain environmental relevance. They stated that "…GLY technical resulted in no mortality at concentrations up to 17.9 mg a.e./L." Hence, the detailed LC50 value remains unknown.

c) Also MANN & BIDWELL (1999) observed no mortality for tadpoles at approximately 400 mg a.e./L.

Scientists have worried about negative effects of GLY use on amphibians for more than a decade and, therefore, have conducted different ecotoxicological experiments with this vertebrate group. Because these experiments did not follow standard protocols and varied in their design (e.g. renewing of the treatment, study duration etc.), results cannot be directly compared among each other and with official tests. However, the experiments provide first insights and are valuable with respect to the amount of data. In the following, the most important laboratory, mesocosm and field studies on the impact of GLY, its formulations and its surfactants on amphibians are summarised and interpreted. Tables 16 to 19 contain ecotoxicological endpoints for acute toxicity (LC50, EC50) to different amphibian life-stages. Endpoints were all determined through laboratory studies. Table 20 overviews the results and main conclusions of studies in the laboratory, but also of mesocosm and field experiments with regard to acute and chronic effects of GLY, its formulations and surfactants as single stressors on different amphibian life-stages.

There are hardly any studies which investigated the effect of AMPA, the major metabolite of GLY, on amphibians. Only an unpublished study by J. NAUGHTON of Warren Wilson College;

Asheville, North Carolina (USA), is available entitled "Response of larval Wood frogs (*Rana sylvatica*) to the herbicide metabolite AMPA (aminomethylphosphonic acid)" (abstract available at http://www.warren-wilson.edu/~research/Undergrad_Res/NSS2010-2011/Abstracts/JakeNaughton.pdf). In this study, tadpoles were exposed to AMPA at concentrations of 0, 40 and 400 µg/L. After a week, total length and tail length growth differed significantly in the three groups. The results indicate that AMPA may affect the growth of larval Wood frogs (as presumably of other amphibians). As mentioned before, acute toxicity studies on freshwater fishes, invertebrates and birds indicate that AMPA is not more toxic than its parent, GLY (CAREY et al. 2008).

Tab. 16: Results of laboratory studies on the acute toxicity of GLY on anuran embryos and larvae
(in alphabetical order).

Species	Life-stage a)	Native to	Test substance	Exposure time [h]	LC50 [mg a.e./L]	References
Crinia insignifera	Tadpoles, Gosner 25	Australia	GLY isopropylamine salt	24, 48	**>400, >400**	MANN & BIDWELL 1999
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	GLY isopropylamine salt	24, 48	**>400, >400**	MANN & BIDWELL 1999
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	GLY isopropylamine salt	24, 96	**>17.9, >17.9**	HOWE et al. 2004
Lithobates pipiens	Tadpoles, 7 days old	Canada, USA	GLY isopropylamine salt	96	**6.5**	TRUMBO 2005
Litoria moorei	Tadpoles, Gosner 25	Australia	GLY isopropylamine salt	24, 48	**>343, >343**	MANN & BIDWELL 1999
Litoria moorei	Tadpoles, Gosner 25	Australia	GLY acid	24, 48	**127.0**, **121.0**	BIDWELL & GORRIE 1995
Litoria moorei	Tadpoles, Gosner 25	Australia	GLY acid	24, 48	**88.6**, **81.2**	MANN & BIDWELL 1999
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Rodeo® (without surfactant, i.e. only GLY isopropylamine salt)	96	**7,298.8**	PERKINS et al. 2000

Legend:
Values cannot be directly compared due to variable test procedures (see text). For detailed information see chapter 5.4.1. Tadpole stages are those of GOSNER (1960).
a) Life-stage at the beginning of the test.

Tab. 17: Results of laboratory studies on the acute toxicity of GBH on amphibian embryos and larvae, orders Anura and Caudata (in alphabetical order).

Species	Life-stage [a)]	Native to	Test substance	Exposure time [h]	LC50 [mg a.e./L]	References
Anura						
Anaxyrus americanus	Embryos	Canada, USA	Vision®	96	**6.4** (at pH 7.0)	EDGINTON et al. 2004
Anaxyrus americanus	Embryos	Canada, USA	Vision®	96	**4.8** (at pH 6.0)	EDGINTON et al. 2004
Anaxyrus americanus	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**2.9** (at pH 6.0)	EDGINTON et al. 2004
Anaxyrus americanus	Embryos	Canada, USA	Vision®	96	**1.7** (at pH 7.0)	EDGINTON et al. 2004
Anaxyrus americanus	Embryos, Gosner 20	Canada, USA	Roundup Original®	24, 96	**>8, 8**	HOWE et al. 2004
Anaxyrus americanus	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	24, 96	**4.2, <4**	HOWE et al. 2004
Anaxyrus americanus	Tadpoles, Gosner 25	Canada, USA	Roundup® formulation [b)]	384	**1.9**	RELYEA 2005a
Anaxyrus americanus	Tadpoles, Gosner 25	Canada, USA	Roundup Original Max®	96	**1.6**	RELYEA & JONES 2009
Anaxyrus boreas	Tadpoles, Gosner 25	Canada, Mexico, USA	Roundup Original Max®	96	**2.0**	RELYEA & JONES 2009
Anaxyrus boreas	Tadpoles, 24h after	Canada, Mexico, USA	Roundup Original®	24, 168, 360	**2.0, 1.6, 1.5**	KING & WAGNER 2010

	hatching					
Anaxyrus fowleri	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	96	**4.2**	FUENTES et al. 2011
Anaxyrus fowleri	Tadpoles, Gosner 25	Canada, USA	Roundup WeatherMAX®	96	**2.0**	FUENTES et al. 2011
Centrolene prosoblepon	Tadpoles, Gosner 25	Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, Panama	Glyfos® + Cosmo-Flux®	96	**2.4**	BERNAL et al. 2009a
Crinia insignifera	Tadpoles, Gosner 25	Australia	Roundup Original®	24, 48	[c], **3.6**	MANN & BIDWELL 1999
Crinia insignifera	Tadpoles, Gosner 25	Australia	Touchdown®	24, 48	**13.1**, **9.0**	MANN & BIDWELL 1999
Dendropsophus microcephalus	Tadpoles, Gosner 25	Belize, Brazil, Colombia, Costa Rica, French Guiana, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Suriname, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96	**1.2**	BERNAL et al. 2009a
Engystomops pustulosus	Tadpoles, Gosner 25	Belize, Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96	**2.8**	BERNAL et al. 2009a
Heleioporus eyrie	Tadpoles, Gosner 25	Australia	Roundup Original®	24, 48	**8.6**, **6.3**	MANN & BIDWELL 1999
Heleioporus eyrie	Tadpoles, Gosner 25	Australia	Touchdown®	24, 48	**16.6**, **16.1**	MANN & BIDWELL 1999

Hyla chrysoscelis	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	96	**2.5**	FUENTES et al. 2011
Hyla chrysoscelis	Tadpoles, Gosner 25	Canada, USA	Roundup WeatherMAX ®	96	**3.3**	FUENTES et al. 2011
Hyla versicolor	Tadpoles, Gosner 25	Canada, USA	Roundup® formulation [b)]	384	**1.0**	RELYEA 2005a
Hyla versicolor	Tadpoles, Gosner 25	Canada, USA	Roundup Original Max®	96	**1.7**	RELYEA & JONES 2009
Hypsiboas crepitans	Tadpoles, Gosner 25	Brazil, Colombia, French Guiana, Guyana, Panama, Suriname, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	9	**2.1**	BERNAL et al. 2009a
Hypsiboas crepitans [d)]	Tadpoles, Gosner 25	Brazil, Colombia, French Guiana, Guyana, Panama, Suriname, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96	**7.3**	BERNAL et al. 2009b
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	Roundup Original®	24, 48	**4.6**, **3.0**	MANN & BIDWELL 1999
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	Touchdown®	24, 48	**14.7**, **12.0**	MANN & BIDWELL 1999
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	Roundup Biactive®	24,48	**>400, >400**	MANN & BIDWELL 1999
Lithobates catesbeianus	Tadpoles, Gosner 25	Canada, Mexico, USA; invasive in different parts of the world	Roundup® formulation [b)]	384	**1.6**	RELYEA 2005a
Lithobates catesbeianus	Tadpoles, Gosner 25	Canada, Mexico, USA; invasive in different parts of the world	Roundup Original Max®	96	**0.8**	RELYEA & JONES 2009

Lithobates catesbeianus	Tadpoles, Gosner 25	Canada, Mexico, USA; invasive in different parts of the world	Roundup Original®	96	**2.8**	FUENTES et al. 2011
Lithobates catesbeianus	Tadpoles, Gosner 25	Canada, Mexico, USA; invasive in different parts of the world	Roundup WeatherMAX®	96	**2.0**	FUENTES et al. 2011
Lithobates clamitans	Embryos	Canada, USA	Vision®	96	**5.3** (at pH 6.0)	EDGINTON et al. 2004
Lithobates clamitans	Embryos	Canada, USA	Vision®	96	**4.1** (at pH 7.0)	EDGINTON et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**3.5** (at pH 6.0)	EDGINTON et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**1.4** (at pH 7.0)	EDGINTON et al. 2004
Lithobates clamitans	Embryos, Gosner 20	Canada, USA	Roundup Original®	24, 96	**>8, 7.1**	HOWE et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	24, 96	**2.0, 2.0**	HOWE et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**2.7 - 4.3**	WOJTASZEK et al. 2004 [e)]
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Roundup® formulation [b)]	384	**1.7**	RELYEA 2005a
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Roundup Original Max®	96	**1.4**	RELYEA & JONES 2009
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Roundup Biactive®	24, 96	**>17.9, >17.9**	HOWE et al. 2004

Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Roundup Transorb®	24, 96	**2.3, 2.2**	HOWE et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Touchdown®	24, 96	**>17.9, >17.9**	HOWE et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Glyfos Bio®	24, 96	**>17.9, >17.9**	HOWE et al. 2004
Lithobates clamitans	Tadpoles, Gosner 25	Canada, USA	Glyfos AU®	24, 96	**9.0, 8.9**	HOWE et al. 2004
Lithobates pipiens	Embryos	Canada, USA	Vision®	96	**15.1** (at pH 6.0)	EDGINTON et al. 2004
Lithobates pipiens	Embryos	Canada, USA	Vision®	96	**7.5** (at pH 7.0)	EDGINTON et al. 2004
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**1.8** (at pH 6.0)	EDGINTON et al. 2004
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Vision®	96	**1.1** (at pH 7.0)	EDGINTON et al. 2004
Lithobates pipiens	Embryos, Gosner 20	Canada, USA	Roundup Original®	24, 96	**>8, 6.5**	HOWE et al. 2004
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	24, 96	**3.7, 2.9**	HOWE et al. 2004
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Roundup® formulation [b)]	384	**1.9**	RELYEA 2005a
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Roundup Original Max®	96	**1.5**	RELYEA & JONES 2009

Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	96	**1.8**	FUENTES et al. 2011
Lithobates pipiens	Tadpoles, Gosner 25	Canada, USA	Roundup WeatherMAX®	96	**2.3**	FUENTES et al. 2011
Lithobates sphenocephalus	Tadpoles, Gosner 25	USA	Roundup Original®	96	**2.1**	FUENTES et al. 2011
Lithobates sphenocephalus	Tadpoles, Gosner 25	USA	Roundup WeatherMAX®	96	**1.3**	FUENTES et al. 2011
Lithobates sylvaticus	Tadpoles, Gosner 25	Canada, USA	Vision®	24	**2.2 - 3.6**	GLASER 1998
Lithobates sylvaticus	Embryos, Gosner 20	Canada, USA	Roundup Original®	24, 96	**>8, >8**	HOWE et al. 2004
Lithobates sylvaticus	Tadpoles, Gosner 25	Canada, USA	Roundup Original®	24, 96	**5.6, 5.1**	HOWE et al. 2004
Lithobates sylvaticus	Tadpoles, Gosner 25	Canada, USA	Roundup® formulation [b)]	384	**1.0**	RELYEA 2005a
Lithobates sylvaticus	Tadpoles, Gosner 25	Canada, USA	Roundup Original Max®	96	**1.9**	RELYEA & JONES 2009
Litoria moorei	Tadpoles, Gosner 25	Australia	Roundup Original®	24, 48	**12.7**, **11.6**	BIDWELL & GORRIE 1995
Litoria moorei	Tadpoles, Gosner 25	Australia	Roundup Original®	24, 48	**3.1**, **2.9**	MANN & BIDWELL 1999
Litoria moorei	Tadpoles, Gosner 25	Australia	Roundup Biactive®	24, 48	**333.0**, **328.0**	MANN & BIDWELL 1999

Litoria moorei	Tadpoles, Gosner 25	Australia	Touchdown®	24, 48	**10.4**, [c]	MANN & BIDWELL 1999
Pseudacris crucifer	Tadpoles, Gosner 25	USA	Roundup Original Max®	96	**0.8**	RELYEA & JONES 2009
Pseudacris regilla	Tadpoles, 24h after hatching	Canada, Mexico, USA	Roundup Original ®	24, 168, 360	**0.3, 0.2, 0.2**	KING & WAGNER 2010
Rana cascadae	Tadpoles, Gosner 25	USA	Roundup Original Max®	96	**1.7**	RELYEA & JONES 2009
Rana cascadae	Tadpoles, 24h after hatching	USA	Roundup Original ®	24, 168, 360	**1.6, 1.1, 1.0**	KING & WAGNER 2010
Rana luteiventris	Tadpoles, 24h after hatching	Canada, USA	Roundup Original ®	24, 168, 360	**1.2, 0.8, 0.7**	KING & WAGNER 2010
Rhinella arenarum	Tadpoles, Gosner 36-38	Argentina, Bolivia, Brazil, Uruguay	Roundup Ultra Max®	6, 12, 24, 48	**5.6, 3.3, 2.4, 2.4**	LAJMANOVICH et al. 2011
Rhinella arenarum	Tadpoles, Gosner 36-38	Argentina, Bolivia, Brazil, Paraguay, Uruguay	Infosato®	6, 12, 24, 48	**49.7, 47.3, 38.8**	LAJMANOVICH et al. 2011
Rhinella arenarum	Tadpoles, Gosner 36-38	Argentina, Bolivia, Brazil, Paraguay, Uruguay	Glifoglex®	6, 12, 24, 48	**96.9, 77.5, 73.8, 73.8**	LAJMANOVICH et al. 2011

Rhinella arenarum	Tadpoles, Gosner 36-38	Argentina, Bolivia, Brazil, Paraguay, Uruguay	C-K Yuyos Fav®	6, 12, 24, 48	**104.3, 84.1, 77.5, 77.5**	LAJMANOVICH et al. 2011
Rhinella granulosa	Tadpoles, Gosner 25	Argentina, Bolivia, Brazil, Colombia, French Guiana, Guyana, Panama, Paraguay, Suriname, Venezuela	Glyfos® + Cosmo-Flux®	96	**2.4**	BERNAL et al. 2009a
Rhinella granulosa d)	Tadpoles, Gosner 25	Argentina, Bolivia, Brazil, Colombia, French Guiana, Guyana, Panama, Paraguay, Suriname, Venezuela	Glyfos® + Cosmo-Flux®	96	**7.2**	BERNAL et al. 2009b
Rhinella margaritifera	Tadpoles, Gosner 25	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Venezuela	Glyfos® + Cosmo-Flux®	96	**1.5**	BERNAL et al. 2009a
Rhinella marina	Tadpoles, Gosner 25	southern Texas, Central America, northern South America and Trinidad and Tobago; invasive in different parts of the world	Glyfos® + Cosmo-Flux®	96	**2.7**	BERNAL et al. 2009a
Rhinella marina d)	Tadpoles, Gosner 25	southern Texas, Central America, northern South America and Trinidad and Tobago; invasive in different parts of the world	Glyfos® + Cosmo-Flux®	96	**6.0**	BERNAL et al. 2009b
Scinax nasicus	Tadpoles, Gosner 25	Argentina, Bolivia, Brazil, Paraguay, Uruguay	Glyfos®	24, 48, 72, 96	**1.7, 1.3, 1.3, 0.9**	LAJMANOVICH et al. 2003

Scinax ruber	Tadpoles, Gosner 25	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96	**1.6**	BERNAL et al. 2009a
Scinax ruber [d]	Tadpoles, Gosner 25	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96	**6.9**	BERNAL et al. 2009b
Spea bombifrons [f]	Tadpoles, Gosner 29-30, grassland site	Canada, Mexico, USA	Roundup WeatherMAX®	48, 216	**2.0, 2.0**	DINEHART et al. 2010
Spea bombifrons [f]	Tadpoles, Gosner 29-30, cropland site	Canada, Mexico, USA	Roundup WeatherMAX®	48, 216	**1.9, 1.7**	DINEHART et al. 2010
Spea multiplicata [f]	Tadpoles, Gosner 29-30, grassland site	Mexico, USA	Roundup WeatherMAX®	48, 216	**2.3, 1.9**	DINEHART et al. 2010
Spea multiplicata [f]	Tadpoles, Gosner 29-30, cropland	Mexico, USA	Roundup WeatherMAX®	48, 216	**2.1, 2.1**	DINEHART et al. 2010

	site					
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Roundup Original®	96	**9.3**	PERKINS et al. 2000
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Rodeo® (without surfactant, i.e. only GLY isopropylamine salt)	96	**7,298.8**	PERKINS et al. 2000
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Vision®	96	**15.6** (at pH 6.0)	EDGINTON et al. 2004
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Vision®	96	**7.9** (at pH 7.0)	EDGINTON et al. 2004
Xenopus laevis	Tadpoles, Gosner 25	Africa south of the Sahara; invasive in different parts of the world	Vision®	96	**2.1** (at pH 6.0)	EDGINTON et al. 2004
Xenopus laevis	Tadpoles, Gosner 25	Africa south of the Sahara; invasive in different parts of the world	Vision®	96	**0.9** (at pH 7.0)	EDGINTON et al. 2004
Caudata						
Ambystoma gracile	Larvae	Canada, USA	Roundup Original Max®	96	**2.8**	RELYEA & JONES 2009
Ambystoma gracile	Larvae, 24h after	Canada, USA	Roundup Original®	24, 168, 360	[g)] **, 1.3, 1.4**	KING & WAGNER 2010

	hatching					
Ambystoma laterale	Larvae	Canada, USA	Roundup Original Max®	96	**3.2**	RELYEA & JONES 2009
Ambystoma macrodacytlum	Larvae, 24h after hatching	Canada, USA	Roundup Original®	24, 168, 360	[g)]**, 1.4, 1.2**	KING & WAGNER 2010
Ambystoma maculatum	Larvae	Canada, USA	Roundup Original Max®	96	**2.8**	RELYEA & JONES 2009
Notophthalmus viridescens	Larvae	Canada, USA	Roundup Original Max®	96	**2.7**	RELYEA & JONES 2009

Legend:
Values cannot be directly compared due to variable test procedures (see text). For detailed information see chapter 5.4.1. Tadpole stages are those of GOSNER (1960).

a) Life-stage at the beginning of the test.
b) RELYEA (2005a: p. 352) only stated that he used “a commercial form of GLY (Roundup)”.
c) Data for which insufficient data were available to calculate an ecotoxicological endpoint.
d) BERNAL et al. (2009b) exposed tadpoles not under laboratory conditions, but in outdoor microcosms with a soil layer. Note that RELYEA (2005b) also added different soil layers, but without any effect.
e) WOJTASZEK et al. (2004) handled larvae not under laboratory conditions, but in volumes of water enclosed from the rest of the studied wetland.
f) DINEHART et al. (2010) tested grassland vs. cropland populations and did not find significantly different sensitivities.
g) No mortality in salamander larvae was observed within the first 24 hours.

Tab. 18: Results of laboratory studies on the acute toxicity of surfactants used in GBH on anuran embryos and larvae (in alphabetical order).

Species	Life-stage [a)]	Native to	Test substance	Exposure time [h]	LC50 [mg/L]	References
Crinia insignifera	Embryos	Australia	Teric GN8 [b)]	140	**6.4**	MANN & BIDWELL 2000
Lithobates clamitans	Tadpoles, stage 25	Canada, USA	POEA	24, 96	**2.4, 2.2**	HOWE et al. 2004
Lithobates pipiens	Tadpoles, 7 days old	Canada, USA	R-11®	96	**1.7**	TRUMBO 2005
Litoria adelaidensis	Embyros	Australia	Teric GN8 [b)]	140	**9.2**	MANN & BIDWELL 2000
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	Teric GN8 [b)]	140	**3.9, 4.6, 4.8, 5.4** [c)]	MANN & BIDWELL 2000
Xenopus laevis	Embryos	Africa south of the Sahara; invasive in different parts of the world	POEA	96	**6.8**	PERKINS et al. 2000

					EC50 [mg/L] [d)]	
Crinia insignifera	Tadpoles, Gosner 25	Australia	Teric GN8 [b)]	48	**2.7** (mild), **3.8** (full)	MANN & BIDWELL 2001
Crinia insignifera	Tadpoles, Gosner 25	Australia	Agral 600 [e)]	48	**2.7** (mild), **3.5** (full)	MANN & BIDWELL 2001
Crinia insignifera	Tadpoles, Gosner 25	Australia	BS 1000 [f)]	48	**5.3** (mild), **6.0** (full)	MANN & BIDWELL 2001
Heleioporus eyrei	Tadpoles, Gosner 25	Australia	Agral 600 [e)]	48	**>10.6** (mild), **3.5** (full)	MANN & BIDWELL 2001
Heleioporus eyrei	Tadpoles, Gosner 25	Australia	BS 1000 [f)]	48	**<10.0** (mild), **25.4** (full)	MANN & BIDWELL 2001
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	Agral 600 [e)]	48	**4.1** (full)	MANN & BIDWELL 2001
Limnodynastes dorsalis	Tadpoles, Gosner 25	Australia	BS 1000 [f)]	48	**<6.0** (mild), **14.3** (full)	MANN & BIDWELL 2001

Litoria moorei	Tadpoles, Gosner 25	Australia	Agral 600 [e)]	48	**4.6** (full)	MANN & BIDWELL 2001
Litoria moorei	Tadpoles, Gosner 25	Australia	BS 1000 [f)]	48	**<11.0** (mild)	MANN & BIDWELL 2001
Rhinella marina	Tadpoles, Gosner 25	southern Texas, Central America, northern South America and Trinidad and Tobago; invasive in different parts of the world	Teric GN8 [b)]	48	**2.8** (mild), **5.1** (full)	MANN & BIDWELL 2001
Rhinella marina	Tadpoles, Gosner 25	southern Texas, Central America, northern South America and Trinidad and Tobago; invasive in different parts of the world	Agral 600 [e)]	48	**2.9** (mild), **5.4** (full)	MANN & BIDWELL 2001
Xenopus laevis	Tadpoles, Gosner 25	Africa south of the Sahara; invasive in different parts of the world	Teric GN8 [b)]	48	**1.1** (mild), **2.8** (full)	MANN & BIDWELL 2001
Xenopus laevis	Tadpoles, Gosner 25	Africa south of the Sahara; invasive in different parts of the world	Agral 600 [e)]	48	**1.2** (mild), **2.3** (full)	MANN & BIDWELL 2001

Legend:
Values cannot be directly compared due to variable test procedures (see text). For detailed information see chapter 5.4.1. Tadpole stages are those of GOSNER (1960).

a) Life-stage at the beginning of the test.
b) 100% nonylphenol polyethoxylate surfactant (NPE).
c) Four independent experiments.
d) MANN & BIDWELL (2001) defined the EC50 values as ‘mild’ or ‘full’ narcosis of the larvae. When a tadpole failed to swim strongly for at least one second after prodding or if it swam in an uncoordinated manner, it was defined to be under ‘mild narcosis’. ‘Full narcosis’ was defined as total lack of activity. Values could not be calculated for all species.
e) 60% NPE and unspecified concentrations of oleic acid and 2-ethyl hexanol.
f) 100% alcohol alkoxylate.

Tab. 19: Results of laboratory studies on the acute toxicity of GLY and GBH on terrestrial life-stages of anurans
(in alphabetical order).

Species	**Life-stage** [a]	**Native to**	**Test substance**	**Exposure time [h]**	**Exposure type**	**LC50**	**References**
Ascaphus truei	Adults	Canada, USA	GLY isopropylamine salt	96	Intraperitoneally administered	**> 2000** mg a.e./L	McCOMB et al. 2008
Centrolene prosoblepon [b]	Juveniles and adults	Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, Panama	Glyfos® + Cosmo-Flux®	96	Over-spraying	**4.5** kg a.e./ha	BERNAL et al. 2009b
Crinia insignifera	Adults	Australia	Technical-grade GLY acid	24, 48	Contaminated water	**89.6**, **83.6** mg a.e./L	MANN & BIDWELL 1999
Crinia insignifera	Metamorphs	Australia	Roundup Original®	24h, 48h	Contaminated water	**88.7**, **51.8** mg a.e./L	BIDWELL & GORRIE 1995
Crinia insignifera	Adults	Australia	Roundup Original®	24h, 48h	Contaminated water	**52.6**, **49.4** mg a.e./L	BIDWELL & GORRIE 1995
Dendrobates truncatus [b]	Juveniles and adults	Colombia	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**>7.4** kg a.e./ha	BERNAL et al. 2009b

Engystomops pustulosus b)	Juveniles and adults	Belize, Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Trinidad and Tobago, Venezuela	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**19.6** kg a.e./ha	BERNAL et al. 2009b
Pristimantis taeniatus b)	Juveniles and adults	Colombia, Panama	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**5.6** kg a.e./ha	BERNAL et al. 2009b
Rhinella granulosa b)	Juveniles and adults	Argentina, Bolivia, Brazil, Colombia, French Guiana, Guyana, Panama, Paraguay, Suriname, Venezuela	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**6.5** kg a.e./ha	BERNAL et al. 2009b
Rhinella margaritifera b)	Juveniles and adults	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Venezuela	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**14.8** kg a.e./L	BERNAL et al. 2009b
Rhinella marina b)	Juveniles and adults	southern Texas, Central America, northern South America and Trindidad and Tobago; invasive in different parts of the world	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**22.8** kg a.e./ha	
Scinax ruber b)	Juveniles and adults	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Trinidad and	Glyfos® + Cosmo-Flux®	96h	Over-spraying	**7.3** kg a.e./ha	BERNAL et al. 2009b

		Tobago, Venezuela					
Caudata							
Dicamptodon ensatus	Adults	USA	GLY isopropylamine salt	96h	Intraperitonally administered	**< 2,000** mg a.e./L	McCOMB et al. 2008
Ensatina eschscholtzii	Adults	Canada, Mexico, USA	GLY isopropylamine salt	96h	Intraperitonally administered	**1,070** mg a.e./L	McCOMB et al. 2008
Plethodon vehiculum	Adults	Canada, USA	GLY isopropylamine salt	96h	Intraperitonally administered	**1,170** mg a.e./L	McCOMB et al. 2008
Taricha granulosa	Adults	Canada, USA	GLY isopropylamine salt	96h	Intraperitonally administered	**1,250, > 2,600** mg a.e./L	McCOMB et al. 2008

Legend:
Values cannot be directly compared due to different test procedures (e.g. renewing of the treatment). Note that further studies with terrestrial life-stages by RELYEA (2005b) and DINEHART et al. (2009) are not part of this table because no endpoints but proportion of death animals have been calculated. For detailed information, see chapter 5.4.1 and 5.4.2.

a) Life-stage at the beginning of the test.

b) BERNAL et al. (2009b) over-sprayed animals in presence of soil and litter.

Tab. 20: Summary of main conclusions of studies on the impact of GLY, its formulations and surfactants as a single stressor on amphibians (in chronological order).

Amphibian species – life-stages	Test substances a)	Study type	Main conclusions b)	References
Lithobates catesbeianus	GBH	Laboratory	• Roundup® can induce DNA damage in tadpoles	CLEMENTS et al. 1997
Crinia insignifera, Heleioporus eyrie, Limnodynastes dorsalis, Litoria moorei – tadpoles, metamorphs and adults	GBH, GLY	Laboratory	• Due to their surfactants, GBH are more toxic to amphibians than the active ingredient GLY itself. • Tadpoles are likely to be more sensitive than older life-stages. • Amphibians are probably species-specific sensitive to different GBH	BIDWELL & GORRIE 1995; MANN & BIDWELL 1999
Lithobates sylvaticus – tadpoles	GBH	Field	• Eggs from treated areas had smaller post-hatch lengths, juvenile lengths and masses and more post-hatch deformities.	GLASER 1998
Crinia insignifera, Litoria adelaidensis, Xenopus laevis – embryos	SURF		• Growth inhibition as assessed by embryo length was the most sensitive indicator of effect in all three species. • *Xenopus* embryos were more sensitive concerning acute toxic effects than embryos of the two Australian species. • Only *Xenopus* embryos displayed indisputable malformations, but teratogenicity indices for the three	MANN & BIDWELL 2000

			species indicated either no or low teratogenicity.	
Xenopus laevis – embryos	GBH, GLY, SURF	Laboratory	• At non-lethal concentrations (i.e. below the $LC50_{96\text{-}h}$) no significant increase of embryo malformations or reduction of their length was observed. • Toxicity of GBH to amphibian embryogenesis is strongly related to the surfactant system.	PERKINS et al. 2000
Crinia insignifera, Heleioporus eyrei, Rhinella marina, Limnodynastes dorsalis, Litoria moorei, Xenopus laevis – tadpoles	SURF	Laboratory	• All species exhibited nonspecific narcosis following exposure of both surfactants. • EC50 values were lower for NPE than for alcohol ethoxylate.	MANN & BIDWELL 2001
Pseudacris triseriata, Lithobates blairi – tadpoles	GBH	Laboratory	• One species was slightly more sensitive than the other. [See also MANN & BIDWELL (1999).] → Due to species-specific mortality GBH can alter not only population dynamics, but also community dynamics. • Older *L. blairi* tadpoles were much more sensitive than younger ones, but this could be related to higher stress due to relatively higher density at the end of the experiment, i.e. an interspecific interaction.	SMITH 2001
Scinax nasicus – tadpoles	GBH	Laboratory	• Toxicity and larval malformations, probably caused by the surfactant POEA, increased with time and herbicide concentration.	LAJMANOVICH et al. 2003
Lithobates pipiens – tadpoles	GBH	Laboratory	• Survival of tadpoles was significantly reduced at concentrations below the calculated EEC.	CHEN et al. 2004

Anaxyrus americanus, Lithobates pipiens, L. clamitans, L. sylvaticus – tadpoles	GBH, GLY, SURF	Laboratory	• Different formulations varied in their toxicity; the surfactant POEA was the most toxic treatment. • Sensitivity of species differed as already indicated by MANN & BIDWELL (1999) and SMITH (2001). • Tadpoles at different Gosner stages showed different sensitivity; older tadpoles were more sensitive. [See also SMITH (2001).] • POEA and two GBH containing POEA significantly reduced growth and metamorphosis rate and prolonged time to metamorphosis. • Due to exposure to POEA and the two GBH containing POE, tail damages and gonadal abnormalities were observed. Sex ratios were not significantly different, but TRβ mRNA expression significantly increased in young tadpoles → disruption of the thyroid axis can be supposed.	HOWE et al. 2004
Anaxyrus americanus, Hyla versicolor, Lithobates pipiens, L. clamitans, L. catesbeianus – tadpoles	GBH	Laboratory	• Survival and growth of *L. clamitans, L. catesbeianus* and *A. americanus* was not affected at one mg a.i./, but significantly reduced at two mg a.i./L.	RELYEA 2004
Lithobates clamitans, L. pipiens – tadpoles	GBH	Field	• *L. clamitans* tadpoles showed higher mean mortality than *L. pipiens* tadpoles with the highest mortality in over-sprayed wetlands. Herbicide application did not reduce survival significantly. However, control mortality	THOMPSON et al. 2004

			was >10%. [See also criticism from RELYEA (2011).] • Mean concentrations in buffered wetlands were significantly lower than in adjacent and over-sprayed wetlands.	
Lithobates clamitans, L. pipiens – tadpoles	GBH	Field	• Highest concentrations caused rapid mortality in nearly all tadpoles, but the calculated EEC and lower concentrations did not significantly reduce larval survival or growth. • Substantially greater tadpole mortality was observed in the pond with higher pH $\rightarrow$ Experimental site (in situ enclosures) and biotic/abiotic factors (i.e. higher pH and suspended sediments) affect toxicity of Vision® concentrations.	WOJTASZEK et al. 2004
Rana cascadae – tadpoles	GBH	Laboratory	• Concentrations below the LC50 significantly affected larval development and metamorphosis: treated larvae metamorphosed earlier. [This is contrary to the findings of HOWE et al. (2004), which observed prolonged time to metamorphosis.] • However, as in the study by HOWE et al. (2004), a decreased size at metamorphosis was observed that could increase predation risk (i.e. reduced fitness). • Mortality may occur mainly upon chronic exposure because no increased mortality has been observed directly after renewal of the treatments. [This is	CAUBLE & WAGNER 2005

			contrary to the statements of e.g. RELYEA (2005b).]	
Anaxyrus americanus, Hyla versicolor, Lithobates catesbeianus, L. clamitans, L. pipiens, *L. sylvaticus* – tadpoles	GBH	Laboratory	• 0.1 and 1 mg a.i./L did not significantly affect survival. • However, no tadpole survived treatments at 5 to 20 mg a.i./L.	RELYEA 2005a
Anaxyrus americanus, A. woodhousii, Hyla versicolor, Lithobates pipiens, L. sylvaticus – tadpoles and juveniles	GBH	Mesocosms	• Added soil did not reduce toxic effects of GBH → Tadpoles rapidly died within 24 hours, before GLY adsorbed to the soil. • 3.8 mg a.i./L significantly reduced survival in all species; only 2% of tadpoles survived after 20 days. • Direct application of 6.5 mL Roundup® Weed & Grass Killer significantly reduced survival of juveniles. Only 21% of the juveniles survived after one day → Amphibian juveniles appear to be very sensitive to terrestrial application of GBH.	RELYEA 2005b
Anaxyrus americanus, Hyla versicolor, Lithobates pipiens, *L. sylvaticus, Pseudacris crucifer* – tadpoles	GBH	Mesocosms	• Roundup® directly affected amphibian diversity rather than indirectly (i.e. via food reduction). • While the tested herbicide 2,4-D had no effect on tadpoles, Roundup® addition completely eliminated tadpoles of two species and nearly of a third one. • Different pesticides can have profound impacts on aquatic communities. The study highlights the importance to examine pesticides within a natural	RELYEA 2005c

			context (e.g. the tested insecticides negatively affected predators and cladocerans and thereby indirectly positive copepods, phytoplankton and tadpoles).	
Lithobates pipiens – tadpoles	GBH, SURF	Laboratory	• Comparable to POEA, the NPE surfactant was more toxic than GLY isopropylamine salt. [However, the author exposed animals to a Rodeo®/R-11® mixture and calculated LC values for the a.i. from these results. Hence, no direct tests with GLY isopropylamine salt took place.]	TRUMBO 2005
Hyla versicolor, H. chrysoscelis – adults	GBH	Field	• Female treefrogs completely avoided breeding ponds when a GBH at 2.4 mg a.e./L was present in the water.	TAKAHASHI 2007
Ascaphus truei, Dicamptodon ensatus, Ensatina eschscholtzii, Plethodon vehiculum, Taricha granulosa – adults	GLY		• GLY isopropylamine salt is practically non-toxic when injected intraperitoneally.	McCOMB et al. 2008
Pelophylax kl. esculentus – ovarian tissue and testis	GLY		• GLY showed no effect on gonadal steroidogenesis.	QUASSINTI et al. 2009
Pelophylax kl. esculentus – skin	GLY		• GLY passes amphibian skin 26 times faster than mammal skin.	QUARANTA et al. 2009
Centrolene prosoblepon, Dendropsophus microcephalus, Engystomops pustulosus, *Hypsiboas*	GBH	Laboratory	• These tropical amphibians were neither more nor less sensitive than amphibians from temperate zones. • The adjuvant added to the ‘coca mixture’ did not increase the toxicity.	BERNAL et al. 2009a

crepitans, Rhinella granulosa, R. marina, R. margaritifera, Scinax ruber – tadpoles			• POEA was supposed mainly responsible for observed mortality. • Most of the toxic responses were expressed within the first two days. • Behavioural responses like slow swimming or remaining on the bottom with no movement (‘clinical signs’) were also observed at low concentrations.	
Centrolene prosoblepon, *Dendrobates truncatus, Engystomops pustulosus, Hypsiboas crepitans, Pristimantis taeniatus, Rhinella granulosa, R. marina, R. margaritifera, Scinax ruber*	GBH	Microcosms and laboratory	• Acute toxicity for tadpoles was approximately similar between species. • In all cases, the spray mixture was less toxic than in the laboratory tests by BERNAL et al. (2009a) and according to the authors this was due to the presence of soil. ([The result is contrary to the findings of RELYEA (2005b).] • In the terrestrial experiment, sensitivity was species-specific. BERNAL et al. (2009b) assumed the thinner skin or the greater surface area to body mass ratio of some species responsible for their higher sensitivity. • Other signs of toxicity included slow movement, extension of the hind limbs and milky secretion from the skin.	BERNAL et al. 2009b
Hyla versicolor, Lithobates pipiens – tadpoles	GLY	Mesocosms	• In this experiment, 10 ppb GLY did not affect tadpole survival, mass at metamorphosis or time to metamorphosis.	RELYEA 2009

Ambystoma gracile, A. maculatum, A. laterale, Anaxyrus americanus, *A. boreas, Hyla versicolor, Lithobates pipiens, L. clamitans, L. sylvaticus, L. catesbeianus, Notophthalmus viridescens, Pseudacris crucifer, Rana cascadae* – larvae	GBH	Laboratory	• $LC50_{96\text{-}h}$ values for larval anurans ranged from 0.8 to 2.0 mg a.e./L whereas $LC50_{96\text{-}h}$ values for larval urodels ranged from 2.7 to 3.2 mg a.e./L→ larval urodels seem to be more resistant to GBH than tadpoles.	RELYEA & JONES 2009
Anaxyrus cognatus, Spea multiplicata – metamorphs	GBH	Laboratory	• One GBH labelled for private use in gardens killed nearly all metamorphs within 48 hours. Another formulation commonly used in gardens killed more than half of toad metamorphs but practically no spadefoot metamorphs when kept on paper towels and was non-toxic when animals were kept on soil. • When the animals were kept on paper towels, several of the tested herbicides had negative effects on survival; when kept on soil, just one of the herbicides impaired their survival → Adsorption to soil particles can intoxicate some formulations while others may kill more rapidly or the relevant substances of these formulations may not be adsorbed fast enough.	DINEHART et al. 2009
Spea multiplicata, S. bombifrons – tadpoles	GBH	Laboratory	• With regard to acute toxicity, both species were more	DINEHART et al. 2010

			sensitive to Roundup WeatherMAX® than to a glufosinate-based herbicide. • Chronic exposure to EEC of Roundup WeatherMAX® caused extensive mortality among larvae of both species, whereas EEC of the glufosinate-based herbicide did not.	
Chioglossa lusitanica – embryos	GBH	Laboratory	• Roundup Plus® did not affect embryo survival, hatching time or developmental stage → Embryos seem to be more resistant than larvae. • Treated animals were larger than controls at hatching.	ORTIZ-SANTALIESTRA et al. 2011
Anaxyrus americanus, Hyla versicolor, Pseudacris triseriata – tadpoles and metamorphs	GBH	Laboratory	• GBH belonged to the most harmful among the tested herbicides. • Environmental relevant concentrations of Roundup WeatherMAX®, as low as national drinking water standards, killed 80% of *P. triseriata* tadpoles. This mortality appears to be associated with the surfactant present in this GBH. • Larval period of *P. triseriata* and *A. americanus* was lengthened by two GBH. [See also HOWE et al. 2004; but contrary: CAUBLE & WAGNER 2005.] This delay could have disproportionate consequences on those amphibian species that oviposit in ephemeral ponds.	WILLIAMS & SEMLITSCH 2010
Anaxyrus americanus, Lithobates sylvaticus	GBH	Mesocosms	• Multiple small doses caused weaker effects than a single large dose of the same total amount.	JONES et al. 2010

			• The impact of a single dose declined with time, probably because tadpoles are more sensitive in their early ontogeny. [This is contrary to the findings of SMITH (2001) and HOWE et al. (2004), which stated higher sensitivity of older tadpoles.] • Observed mass reduction upon herbicide application was not because less food (i.e. periphyton) was available, but because the herbicide altered the tadpole's behaviour or physiology. • Herbicide concentrations were considerably higher near the surface → Ectotherme amphibian larvae typically seek the warmer temperatures of surface waters.	
Xenopus laevis – embryos	GBH, GLY	Laboratory	• Roundup Classic® and GLY itself caused malformations in frog and chicken embryos. • Malformations observed in the offspring of humans that live in areas of intensive GLY use are principally the same as in frog embryos. • [However, high concentrations (72 mg a.e./L) of the GBH were used and GLY was directly injected.]	PAGANELLI et al. 2010
Rhinella arenarum – tadpoles	GBH	Laboratory	• Tadpoles showed different sensitivity to the tested GBH. • Formulations inhibited enzymes involved in metabolism and detoxification. → Depletion of certain	LAJMANOVICH et al. 2011

	enzymes (glutathione S-transferases) leads to oxidative stress, which could be part of the toxicity mechanism of GBH.

Legend
Note that different concentrations, test substances and study designs were used (see text). For an overview of studies on interactions with further stressors, see Table 21.

[a] SURF = surfactant(s)

[b] Cross references in brackets are not part of the study conclusions, but provided to help understanding.

5.4.1 Laboratory studies

The most important laboratory studies on effects of GLY and its formulations are summarised in chronological order below and loosely grouped by subheadings. Main conclusions of these laboratory studies are summarised in Table 20. Interim conclusions of laboratory, mesocosm and field studies can be found at the end of chapter 5.4. Further laboratory studies investigated interactions of GLY or GBH with other stressors. Results from these studies can be found in chapter 5.6, whereas here only effects of GLY, its formulations and surfactants as a single stressor are dealt with. Some study details like tested concentrations, exposure time etc. are given for comparative reasons. All experiments used at least three replicates for each tested concentration.

First studies on GLY and GBH on amphibians

BIDWELL & GORRIE (1995) were the first to study effects of GLY and some of its formulations on amphibians. Procedures were principally the same as in MANN & BIDWELL (1999; p. 194-195; see below) and the results of both studies were published together because BIDWELL & GORRIE (1995) only produced an unpublished report for the Australian Department of Environmental Protection. That is why the study of MANN & BIDWELL (1999) was the first widely recognised study on acute toxic effects of GLY and its formulations on amphibians. Both studies compared the LC50 values of technical-grade GLY acid, GLY isopropylamine salt (i.e. only the active ingredient) and three GBH[19] on four Australian frog species[20]. This was accomplished by exposing tadpoles of all four species at Gosner stage 25 (cf. GOSNER 1960) and, in addition, metamorphs and adults of the Sign-bearing frog (*Crinia insignifera*) to the various substances for 24 to 48h. When range-finding indicated no mortality at or above 400 mg a.e./L, tests were restricted to a single concentration of 400 mg a.e./L[21]. The authors found a considerably higher acute toxicity of the formulations than of the active ingredient to Australian frogs (see Tables 16-17). Because Roundup Original® was the most toxic substance[22], the authors presumed that the surfactant POEA was the primary agent responsible for toxicity. Although there was no clear trend, species-specific sensitivity to different formulations could be observed. With regard to different life-stages, adults and metamorphs of *Crinia insignifera* were much less sensitive to Roundup Original® than its tadpoles (although differing test conditions make this comparison tenuous).

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[19] Roundup Original®, Roundup Biactive® and Touchdown®.

[20] The four test species are commonly found in southwestern Australian agricultural areas: the Sign-bearing frog (*Crinia insignifera*), the Moaning frog (*Heleioporus eyrie*), the Western bullfrog (*Limnodynastes dorsalis*) and the Bell frog (*Litoria moorei*).

[21] Therefore, LC50 values could not be calculated for all species and life-stages. For example, glyphosate isopropylamine salt was practically non-toxic at the applied concentrations and so LC50 values could not be calculated for several species.

[22] Followed by Touchdown®, technical-grade glyphosate acid, Roundup Biactive® and glyphosate isopropylamine salt (see Tables 16 and 17).

The first study on the genotoxicity of Roundup® was conducted by CLEMENTS et al. (1997). They exposed American bullfrog (*Lithobates catesbeianus*) tadpoles to four concentrations[23]. After exposure, DNA damages in erythrocytes were studied using a standard method ('comet assay'; see SINGH et al. 1988). All tadpoles in the highest concentration group died and were excluded from further analysis. Compared to the control group, no significant DNA damages were observed at the lowest concentration, but at 6.75 and 27 mg/L a dose-response relationship emerged. When testing four other herbicides, CLEMENTS et al. (1997) received also dose-response relationships with two of them (AAtrex Nine-O® including atrazine, and Dual-960E® including metolachlor), while the others induced DNA damage but without a dose-response (Sencor-500F® including metribuzin) or no significant DNA damage at all (Amsol including 2,4-D amine). CLEMENTS et al. (1997) concluded that use of some herbicides including Roundup® is capable of inducing DNA damage in tadpoles when concentrations in the water are high enough. However, consequences of such DNA damages on the health of individuals or their offspring remain unclear.

Besides her field study (see chapter 5.4.3), GLASER (1998) conducted a laboratory study on the acute toxicity of the GBH Vision®[24] to Wood frog (*Lithobates sylvaticus*) tadpoles. Treatments were renewed daily. All tadpoles in 0 and 2.2 mg a.e./L survived the 96h experiment, while tadpoles in all remaining groups died within the first 24h. GLASER (1998) stated that because tadpole response "jumped from 0 to 100% mortality from 2.2 to 3.6 mg GLY a.e./L, an LC50 could not be calculated" but that the $LC50_{24\text{-h}}$ "should lie between" the values mentioned.

GBH and surfactants

PERKINS et al. (2000) employed the standard FETAX ('Frog Embryo Teratogenesis Assay – *Xenopus*')[25] to evaluate the effects of two GBH (Roundup Original®, Rodeo®) and of the POEA surfactant alone on the embryogenesis of the African clawed frog (*Xenopus laevis*). Embryos at the blastula stage were exposed to the chemicals at different concentrations for 96h while developing into free swimming larvae. Embryo mortality at various concentrations was used to determine the $LC5_{96\text{-h}}$, $LC10_{96\text{-h}}$ [26] and $LC50_{96\text{-h}}$ values (for the $LC50_{96\text{-h}}$ values see Table 16). According to the results, the surfactant POEA was more toxic than Roundup Original® and this was much more (700 fold) toxic than Rodeo®. At non-lethal concentrations none of the substances significantly increased embryo malformations or reduction of their length. The main conclusion of this study was that toxicity of GBH to amphibian embryogenesis is strongly related to the surfactant system.

23 1.69, 6.75, 27 and 108 mg/L, each for 24h.
24 Concentrations of 0, 2.2, 3.6, 4.3 and 5.7 mg a.e./L.
25 FETAX is a 96 hour whole embryo assay for identifying teratogenic and developmental toxicants. The latter are defined as "as (a) substance(s) that result(s) in embryo mortality, malformation, or growth inhibition at concentrations far less than those required to affect adult organisms" (AMERICAN SOCIETY FOR TESTING AND MATERIALS 1992). The assay is called 'static renewal', because solutions are renewed every 24 hours.
26 $LC5_{96\text{-h}}$: 5,515.5 mg a.e./L for Rodeo®, 7.7 mg a.e./L for Roundup® and 5.8 mg a.e./L for POEA.

MANN & BIDWELL (2000) used the FETAX protocol to assess the teratogenicity of nonylphenol ethoxylate (NPE), a surfactant partly used in GBH. Besides the African clawed frog, embryos of two Australian species[27] were tested. Growth inhibition as assessed by embryo length was the most sensitive indicator of effect in all three species. Embryos of the African clawed frog were more sensitive concerning acute toxic effects than embryos of the two Australian species (see Table 18). The African clawed frog was the only species that displayed indisputable malformations. However, teratogenicity indices for the three species indicated either no or low teratogenicity.

MANN & BIDWELL (2001) exposed Gosner stage 25 tadpoles (cf. GOSNER 1960) of the African clawed frog, four Australian species[28] and the Cane toad (*Rhinella marina*) to different surfactants[29] that are partly used in GBH to investigate endpoints of acute toxicity. 'Full' and 'mild' narcosis, respectively, were considered as half maximal effective concentration (EC50)[30]. All species exhibited nonspecific narcosis following exposure of both surfactants. $EC50_{48\text{-}h}$ values were lower for NPE than for alcohol ethoxylate (Table 18). Narcotic effects were not particularly time dependent when Cane toad tadpoles were exposed to the surfactants at higher temperature (30°C). Lower oxygen content produced a two to threefold increase in toxicity. Together with higher temperatures both can be seen as further stressors, which usually prevail in shallow lentic ponds (see also chap. 5.6).

North and South American anurans

In the first recognised study on North American anurans[31], SMITH (2001) tested whether Kleeraway®, a GBH registered for domestic and agricultural use[32], was acute toxic to tadpoles and whether exposure affects their growth and development. First, Gosner stage 25 to 30 tadpoles (cf. GOSNER 1960) were exposed to different test concentrations[33] for 24h. Then surviving tadpoles were placed into freshwater to control their growth and development for two more weeks. Mortality in *Pseudacris triseriata* was higher than in *Lithobates blairi*: No *P. triseriata* tadpole survived the higher test concentrations and only less than half of them survived the lowest one. Furthermore, all *L. blairi* tadpoles died at the highest concentration and all older larvae in the lowest concentration whereas all young *L. blairi* tadpoles survived the lowest concentration. SMITH (2001) stated the species-specific sensitivity already supposed by MANN & BIDWELL (1999) and concluded that

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$LC10_{96\text{-}h}$: 5,867.2 mg a.e./L for Rodeo®, 8.0 mg a.e./L for Roundup® and 6.0 mg a.e./L for POEA.

27 *Litoria adelaidensis* and *Crinia insignifera.*

28 *Crinia insignifera, Heleioporus eyrei, Limnodynastes dorsalis and Litoria moorei.*

29 Nonylphenol ethoxylate (NPE) and alcohol alkoxylate.

30 MANN & BIDWELL (2001) defined the EC50 values as 'mild' or 'full' narcosis of the larvae. When a tadpole failed to swim strongly for at least one second after prodding or if it swam in an uncoordinated manner, it was defined to be under 'mild narcosis'. 'Full narcosis' was defined as total lack of activity. Values could not be calculated for all species.

31 Western chorus frog (*Pseudacris triseriata*) and Plains leopard frog (*Lithobates blairi*).

32 This formulation does not contain POEA, but an ethoxylated tallowamine surfactant.

33 Control, 0.1 concentration, i.e. 1 part Kleeraway® to 9 parts of water, 0.01 concentration, 0.001 concentration and 0.0001 concentration.

GBH may alter not only population dynamics, but also community dynamics via differences in species-specific mortality rate. SMITH (2001) suggested that the higher mortality of older *L. blairi* tadpoles was related to higher stress due to higher relative density in the aquaria, i.e. due to their larger size, and not because of any differences in sensitivity of different life-stages. No impact of the GBH on further growth and development of the tadpoles could be observed. Although four of nine *P. triseriata* tadpoles previously treated with the lowest concentration died within the first two days in the subsequent growth and development study (all *L. blairi* survived), final mass and development of the survivors did not significantly differ from the control. Hence, SMITH (2001) did not observe chronic effects of sublethal concentrations.

The first study on a South American amphibian species is that of LAJMANOVICH et al. (2003). It examined the acute toxicity of Glyfos®[34] to tadpoles at Gosner stages 25-26 (cf. GOSNER 1960) of the treefrog *Scinax nasicus* up to 96h[35]. Solutions were renewed daily. Mortality was elevated with increasing GLY concentration and prolonged exposure time (dose-response). In addition, larval malformations occurred and increased in the same manner. The gills of *S. nasicus* tadpoles were especially sensitive. This has been observed for other herbicides, too (LAJMANOVICH et al. 1998; see chapter 5.5.1). LAJMANOVICH et al. (2003) supposed that the surfactant (i.e. POEA) rather than the active ingredient of the formulation may be responsible for the observed larval malformations.

LC values from studies with co-stressors

CHEN et al. (2004) studied the multiple stress effects of Vision®, pH and food on zooplankton and Northern leopard frog (*Lithobates pipiens*) tadpoles, both singly and in combination (for details on the observed interactions, see chapter 5.6.2.1). Gosner stage 25 (cf. GOSNER 1960) tadpoles were exposed to two concentrations of Vision®[36] over 10 days with daily renewed solutions. The two tested concentrations were lower or slightly higher than the calculated worst-case value for the Expected Environmental Concentration (EEC = 1.40 mg a.e./L)[37]. Survival of the tadpoles was significantly reduced at both concentrations. CHEN et al. (2004) concluded that Vision® can have significant effects on survival and development at concentrations below the calculated EEC when longer exposed. In the same issue of the journal, THOMPSON et al. (2004) and WOJTASZEK et al. (2004) published their opposing findings from field studies on the impact of Vision® on Northern leopard (*L. pipiens*) and Green frog (*Lithobates clamitans*) larvae (cf. chapter 5.4.3).

RELYEA (2004) conducted a study on the impacts of four different pesticides[38] alone (1-2

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[34] A commercial glyphosate formulation containing 48% glyphosate isopropylamine salt and POEA as surfactant.

[35] Concentrations of 3.07, 3.84, 4.8, 6 and 7.5 mg formulation/L were used.

[36] 0.75 and 1.5 mg a.e./L.

[37] EEC calculated by BOUTIN et al. (1995) assuming full deposition of the maximum label rate into a water body 15 cm in depth.

[38] Three broad-spectrum insecticides, diazinon, malathion and carbaryl, and glyphosate as Roundup®. Unfortunately, RELYEA (2004) did not state the exact name of the glyphosate formulation.

mg a.i./L) or in a pairwise combination (1 mg a.i./L of each pesticide) on Gosner stage 25-tadpoles (see GOSNER 1960) of five North American amphibian species (cf. chapter 5.6.2.1). With regard to the tested Roundup® formulation, 1 mg a.i./L did not negatively impact either survival or growth of any of the tested amphibian species[39], but 2 mg a.i./L caused significant mortality and growth reduction in *L. clamitans, L. catesbeianus* and *A. americanus* tadpoles.

More GBH and surfactants

HOWE et al. (2004) compared the acute toxicity of the active ingredient GLY isopropylamine salt, six different GBH[40] and the surfactant POEA alone. Although these authors collected eggs from four North American anurans[41], only larval *Lithobates clamitans* were exposed to all formulations. For the remaining amphibian species, only the $LC50_{24\text{-}h}$ and the $LC50_{96\text{-}h}$ for Roundup Original® were determined (Table 17). HOWE et al. (2004) used tadpoles at Gosner stages 20 and 25. The different treatments varied in their toxicity to *L. clamitans* tadpoles. POEA alone was about six times more toxic than the most toxic formulation (Roundup Original®), but similarly toxic when the data were compared on the basis of acid equivalent concentrations. HOWE et al. (2004) concluded that environmentally relevant concentrations (as stated by GIESY et al. 2000) of formulations containing POEA can be acutely toxic for the larval stages of these four species. Another main finding was that tadpoles at different Gosner stages showed different sensitivity to exposure (see Table 17). Older larvae were more sensitive, comparable to the results of SMITH (2001), but HOWE et al. (2004) concluded a life-stage dependent sensitivity. Furthermore, *L. clamitans* was the most sensitive and *L. pipiens* the most tolerant species, suggesting that sensitivity of species differed as before stated by MANN & BIDWELL (1999) and SMITH (2001). In addition to acute toxicity, HOWE et al. (2004) investigated chronic exposure to GBH[42] and assessed tadpole morphometrics, tail damage, gonadal histology, sex ratio and gene expression 28 days after the termination of exposure. POEA, Roundup Original® and Roundup Transorb® (both containing POEA) significantly reduced growth and metamorphosis rate in *L. pipiens*. Tadpoles showed smaller size at metamorphosis and took longer to metamorphose. In addition, they sustained tail damage and gonadal abnormalities (but not significantly different to the control) when exposed to both concentrations of the three tested substances (cf. chapter 5.5).

RELYEA (2005a) investigated the impact of a certain Roundup® formulation[43] on six North

[39] The Northern leopard frog (*Lithobates pipiens*), the Green frog (*L. clamitans*), the American bullfrog (*L. catesbeianus*), the American toad (*Anaxyrus americanus*) and the Gray treefrog (*Hyla versicolor*).

[40] Roundup Original®, Roundup Transorb®, Roundup Biactive®, Glyfos AU®, Glyfos BIO® and Touchdown 480®.

[41] *Lithobates clamitans, L. pipiens, L. sylvaticus* and *Anaxyrus americanus.*

[42] HOWE et al. (2004) cultured *Lithobates pipiens* tadpoles from Gosner stage 25 to 42 (cf. GOSNER 1960) and exposed them to 0.6 and 1.8 mg a.e./L for 42 days. Once a week, solutions were renewed.

[43] Unfortunately, RELYEA (2005a) did not state the exact name of the glyphosate formulation. The results of this study could be referred to Roundup Original®, although the author used Roundup Weed & Grass Killer® in his mesocosm study (RELYEA 2005b).

American amphibian species[44]. Besides a long-term acute toxicity testing (16 days), he also investigated the impact of predator cues on larval survival (cf. chapter 5.6.1.2). Because predator cues only affected survival of Wood frog larvae[45], the remaining $LC50_{216\text{-}h}$ values for the other species can be considered as results of ordinary acute toxicity testing[46]. No tadpole survived 5 to 20 mg a.i./L (~ 3.75 and 15 mg a.e./L) treatments with Roundup®. In contrast, at 0.1 and 1 mg a.i./L (~ 0.075 and 0.75 mg a.e./L), survival did not significantly differ to control groups.

TRUMBO (2005) investigated the toxicity of the herbicide Rodeo® and the surfactant R-11® which are commonly mixed to control vegetation in or near surface water. The active ingredients are GLY isopropylamine salt in Rodeo® and the surfactant NPE in R-11®. Rodeo® and R-11® were employed in different mixtures[47] to seven days old tadpoles of the Northern leopard frog (*Lithobates pipiens*). $LC50_{96\text{-}h}$ values for the active ingredients were calculated from the employed products and reported as 6.5 mg a.e./L for GLY isopropylamine salt (Table 16) and 1.7 mg/L for NPE. Hence, the surfactant was more toxic than the herbicidal agent, which is similar to POEA compared to GLY. Special attention should be paid to breakdown products: TRUMBO (2005) mentioned that nonylphenol has been identified as endocrine disruptor and is more toxic than its parent compound NPE. However, this aspect was not further studied. The calculated $LC50_{96\text{-}h}$ value of 6.5 mg a.e./L for GLY is the lowest value reported so far (Table 16). Since Rodeo® but not GLY was actually tested, this value should be treated with some caution. TRUMBO (2005) also employed Rodeo® and R-11® single and in a 2:1 mixture on two fish species and one crustacean species. When Rodeo® was added to R-11®, the toxicity of the surfactant changed little, but the toxicity of the herbicidal agent GLY increased dramatically.

Chronic effects and different exposures

CAUBLE & WAGNER (2005) investigated the chronic exposure of Roundup Original® to Cascade frog (*Rana cascadae*) tadpoles at non-acute levels[48]. Thereby, chronic effects on metamorphosis were examined. Larvae were checked daily for mortality, feeding, swimming activity, abnormalities and metamorphosis stage. The low 1 ppm concentration of Roundup Original® significantly affected larval development and metamorphosis and reduced survivability, rate of metamorphosis and post-metamorphosis mass. At 2 ppm no tadpole survived until metamorphosis with a mean survival time of 7.5 days. At both concentration levels, mortality was not altered when treatment solutions were renewed every 7 days. CAUBLE & WAGNER (2005) concluded that mortality would mainly occur due to chronic exposure. This suggestion is contrary to the statements by e.g.

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[44] *Anaxyrus americanus, Hyla versicolor, Lithobates catesbeianus, L. clamitans, L. pipiens* and *L. sylvaticus*.
[45] Here, predator presence reduced the $LC50_{216\text{-}h}$ value to 0.41 mg a.e./L.
[46] RELYEA (2005a) treated tadpoles at stage 25 (following larval staging of GOSNER 1960) to five different concentrations of the used Roundup® formulation[46]. Solutions were renewed every four days and the experiment was terminated after 16 days.
[47] Rodeo®/R-11® mixtures were 17.6 and 4.5 mg/L; 8.7 and 2.5 mg/L; 4.5 and 1.0 mg/L; 2.4 and 0.6 mg/L; 1.3 and 0.3 mg/L.
[48] Dilution concentrations were 1 and 2 ppm of Roundup Original® because the formerly determined

RELYEA (2005b) or BERNAL et al. (2009a) who observed relatively fast mortality within the first 1-2 days. CAUBLE & WAGNER (2005) found that treated individuals (1 ppm) metamorphosed significantly earlier (about one week) and had a significantly lower body mass than the control group. The authors supposed that in the presence of sublethal Roundup® concentrations larvae metamorphosed earlier due to the suboptimal conditions and would have a higher predation risk due to decreased size in the wild.

McCOMB et al. (2008) injected technical GLY isopropylamine salt intraperitoneally to nine terrestrial vertebrate species (five amphibians[49] and four mammals) and compared LD50 values with those of laboratory mice. Median lethal doses ranged from 800 to 1,340 mg/kg for mammals, and from 1,170 to >2,000 mg/kg for amphibians. The Tailed frog (*Ascaphus truei*) was the least sensitive species (LD50 >2,000 mg/kg) and the mammal Oregon vole the most sensitive one. White lab mice were in the middle of the mammalian range. Some intraperitoneally injected newts were radio-tracked and released. No effects on their movement, foraging or shelter were observed. The authors concluded a large margin of safety between dosages encountered via aerial application of GLY at maximum rates in forests and those causing either death or limitation of movement, foraging or shelter. However, the authors only tested the active ingredient and not formulation including surfactants.

QUARANTA et al. 2009 measured the percutaneous passage of three herbicides (atrazine, paraquat and GLY) in the skin of the waterfrog body and in pig ear. All herbicides passed the frog skin much faster than the pig skin namely 302 times for atrazine, 29 times for paraquat and 26 times for GLY. When herbicides were compared to each other, atrazine passed about 134 times faster than GLY in frog skin, but only 12 times in pig ear skin.

Endocrine effects

QUASSINTI et al. (2009) incubated ovarian tissue and testis of the waterfrog in vitro in presence of different concentrations of GLY and the herbicide paraquat. 17β-estradiol and testosterone levels were measured in the incubation medium by radioimmunoassay. Paraquat acts on gonadal steroidogenesis through a mechanism involving reactive oxygen species. GLY showed no effect on gonadal steroidogenesis. In contrast to this, HOWE et al. (2004) observed potential endocrine effects when using POEA and formulations with POEA.

Coca plantations and soil

The spraying of illicit coca plantations in Colombia with GBH has raised concern about possible impacts on amphibians. Therefore, BERNAL et al. (2009a) examined the acute toxicity of the applied spray mixture containing the GBH Glyfos®, the adjuvant Cosmo-Flux® and POEA as

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$LC50_{48\text{-}h}$ was 3.2 ppm.

49 *Ascaphus truei, Dicamptodon ensatus, Ensatina escholtzii, Plethodon vehiculum* and *Taricha granulosa.*

surfactant[50] using field collected frog eggs[51]. Test concentrations ranged from 1 to 4.2 mg a.e./L, test solutions were renewed daily, and the experiment was terminated after 96 hours. Species showed different sensitivity towards the spray mixture (Table 17). A species sensitivity distribution with data from the literature showed that these Colombian amphibians were neither more nor less sensitive than amphibians from other parts of the world. The adjuvant Cosmo-Flux® did not increase toxicity and therefore BERNAL et al. (2009a) supposed that the surfactant POEA was mainly responsible for observed mortality. Most tadpoles died within the first two days. Behavioural responses like slow swimming or remaining on the bottom with no movement were also observed at low concentrations. BERNAL et al. (2009a) did not evaluate the findings concerning real-world scenarios, but referred to a related article where soil was added to the aquaria. In it, BERNAL et al. (2009b) exposed tadpoles of four Colombian amphibians[52] in six small plastic pools including a layer of local soil to the 'coca-spray-mixture' (i.e. Glyfos® with Cosmo-Flux®). Pools with tadpoles at Gosner stage 25 were over-sprayed with the 'coca-spray-mixture' at rates from 0 to 29.5 kg a.e./ha. Furthermore, juveniles of eight Colombian amphibians[53] were raised under laboratory conditions, and adults of these test species collected in the wild. Plastic tubs were filled with moistened soil and leaf litter. Animals were over-sprayed with a geometric series lower and higher than the field application rate of 3.69 kg a.e./ha. $LC50_{96\text{-}h}$ values for tadpoles were approximately similar and in all cases sensitivity was less than in the laboratory tests by BERNAL et al. (2009a) (see Table 17 and Fig. 12).

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[50] BERNAL et al. (2009a) field-collected eggs from various frog species. Larvae hatched under laboratory conditions and were used for experiments at Gosner stage 25. Furthermore, stage-25-tadpoles of one species, *Scinax ruber,* were collected directly from the field and extended the amount of test species.

[51] It remains unclear if the parental animals or the eggs could be exposed to the spray mixture at the sample locations.

[52] *Hypsiboas crepitans, Rhinella granulosa, R. marina* and *Scinax ruber.*

[53] *Dendrobates truncatus, Rhinella granulosa, R. marina, R. margaritifera, Centrolene prosoblepon*, *Engystomops pustulosus, Pristimantis taeniatus* and *Scinax ruber.*

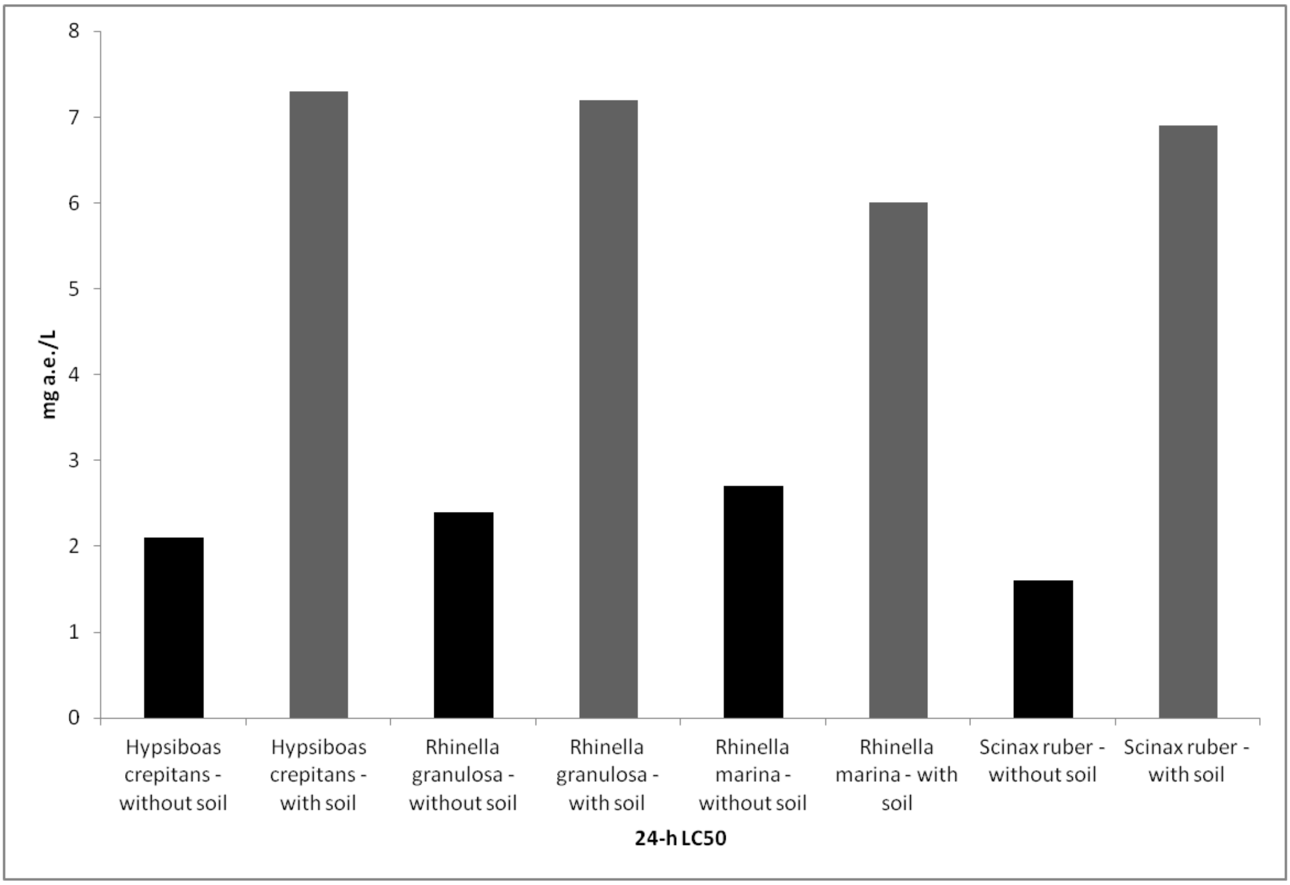

Fig. 12: Apparently positive effects of added soil to tadpoles of some Colombian anurans after exposure to Glyfos® (POEA surfactant + Cosmo-Flux adjuvant)
(data from BERNAL et al. 2009a,b).
Note that only one replicate was used in the 'soil-study' and the water's pH was lower than in the laboratory study. Hence, the comparison should be viewed with caution.

Therefore, BERNAL et al. (2009b) supposed the presence of soil responsible for the observed lower toxicity. This is contrary to the findings by RELYEA (2005b) where addition of soil had no positive effect on survival (see chapter 5.4.2). However, BERNAL et al (2009b) only used one replicate per concentration (six plastic pools) and the average pH (about 7) was lower than in the laboratory experiments (8.2) by BERNAL et al. (2009a). Lower pH leads to higher survival in tadpoles (CHEN et al. 2004; EDGINTON et al. 2004; WOJTASZEK et al. 2004; see chapter 5.6.2.1 for detailed information).

In the terrestrial experiment, sensitivity was also species-specific and up to 30% of the over-sprayed individuals died. Besides direct toxicity, other signs of toxicity included slow movement, extension of the hind limbs and milky secretion from the skin. BERNAL et al. (2009b) assumed the thinner skin or the greater surface area to body mass ratio of some species responsible for their higher sensitivity. The authors concluded that the 'coca-spray-mixture' would have a slight but not an ecologically significant risk to the Colombian amphibian fauna because responses of amphibians to GLY applications under real-world scenarios would be less than what can be predicted from laboratory studies due to e.g. additional vegetation buffers. However, because the study found up to 30% mortality in over-sprayed individuals at recommended application rates, this conclusion of environmental safety seems disputable (see also detailed criticism from RELYEA 2011 on the study design and the conclusions).

Larval urodels

RELYEA & JONES (2009) conducted tests to estimate LC50 values of the GBH Roundup Original Max® containing an unknown surfactant for 13 species of Nearctic amphibians. The test species were of five different amphibian families from both eastern and western North America including nine larval anuran species[54] at Gosner stage 25 (cf. GOSNER 1960). For the first time, acute toxicity testing of a GBH was also conducted using four species of larval urodels[55].[56]. Herbicide exposure had a significant effect on all nine anurans. For American bullfrog and Spring peeper (*Pseudacris crucifer*) larvae, 1 mg a.e./L caused significantly greater mortality than the control; for the remaining seven anurans the parallel concentration was 2 mg a.e./L. $LC50_{96\text{-}h}$ values for the anuran species ranged from 0.8 to 2.0 mg a.e./L (see Table 17). For the larval urodels, only LC50 values could be estimated which were all similar (see Table 17). As a main conclusion, larval urodels seem to be more resistant than tadpoles and Roundup Original Max® with an unknown surfactant (not POEA) was 'highly toxic' (0.1 mg/L<LC50<1 mg/L; as defined by the USEPA: http://www.epa.gov/espp/litstatus/effects/redleg-frog/.) to tadpoles of two species. Hence, not only POEA seems to be responsible for adverse effects but also other surfactants.

Comparisons with other broad-spectrum herbicides

DINEHART et al. (2009) compared the acute toxicity of a glufosinate-based herbicide[57] and three GBH[58] when sprayed over metamorphs of two North American anurans, which are common to sites surrounded by agricultural use[59]. Recently metamorphosed specimens were field-collected from localities in agricultural areas. Therefore, these animals likely had experienced pesticide exposure. Test tubs contained either moistened sandy loam soil from an area where no pesticides have been applied for at least five years or moistened paper towels. All herbicides were applied at the maximum rate allowed for a single application[60]. New Mexico spadefoot (*Spea multiplicata*)

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[54] The ranid species *Lithobates pipiens, L. clamitans, L. sylvaticus, L. catesbeianus, Rana cascadae*, the two bufonid species *Anaxyrus americanus* and *A. boreas* and the two hylid species *Hyla versicolor* and *Pseudacris crucifer*.

[55] Northwestern salamander (*Ambystoma gracile*), Spotted salamander (*A. maculatum*), Blue-spotted salamander (*A. laterale*) (all in family Ambystomatidae) and the Eastern newt (*Notophthalmus viridescens*, family Salamandridae).

[56] For all $LC10_{96\text{-}h}$ and $LC90_{96\text{-}h}$ values, see RELYEA & JONES (2009, page 2007). All animals were collected from natural ponds as newly oviposited eggs. To estimate $LC10_{96\text{-}h}$, $LC50_{96\text{-}h}$ and $LC90_{96\text{-}h}$, all larvae were exposed to 0, 1, 2, 3, 4 and 5 mg a.e./L using static renewal in two types of containers (tadpoles in plastic tubs, larval urodels in petri dishes).

[57] Ignite 280 SL®.

[58] Roundup WeatherMAX®, Roundup Weed & Grass Killer Super Concentrate® and Roundup Weed & Grass Killer Ready-To-Use Plus®.

[59] These were the Great Plains toad (*Anaxyrus cognatus*) and the New Mexico spadefoot (*Spea multiplicata*).

[60] Roundup Weed & Grass Killer Ready-To-Use Plus® 1.33 mL glyphosate/m^2; Roundup Weed & Grass Killer Super Concentrate® 1.33 mL glyphosate/m^2; Roundup WeatherMAX® 0.16 mL glyphosate/m^2;

survival was higher on soil than on paper towels. No spadefoot metamorph survived the exposure to Roundup Weed &Grass Killer Ready-To-Use Plus®, neither on soil nor on paper towels. Conversely, all animals survived the exposure to the remaining herbicides when kept on soil. On paper towels, the other herbicides killed 2.8% to 40.4% of spadefoot metamorphs (see Fig. 13). It is noteworthy that also 28.9% of all animals from the control group housed on paper towels died within 48h. Because usually more than 90% of the control should survive to obtain reliable results (see e.g. RELYEA 2011), these results (*S. multiplicata*) of DINEHART et al. (2009) should be viewed with caution. Survival of Great Plains toad (*Anaxyrus cognatus*) metamorphs was significantly affected by herbicide formulations on each substrate. All Great Plains toad metamorphs exposed to Roundup Weed & Grass Killer Ready-To-Use Plus® on paper towel died, as did 77.5% of those kept on soil. In contrast, all individuals survived exposure to the remaining herbicides when kept on soil. While Ignite 280 SL® killed a similar ratio of toads to spadefoots on paper towels, in other cases, a species-specific sensitivity could be observed. For example, Roundup Weed & Grass Killer Super Concentrate® killed 52.5% of the toads on paper towels, much more than spadefoots. While Roundup WeatherMAX® killed over 40% of spadefoots on paper towels, only 12.5% of toads kept on paper towels died due to exposure to this formulation (see Fig. 13). Mortality in the toad control group was only 2.5%, and, therefore, these results are more reliable than for the spadefoots with 28.9% mortality in the control. To sum up, Roundup Weed & Grass Killer Ready-To-Use Plus® was the most toxic formulation, killing all spadefoot metamorphs and nearly all toad metamorphs, independent of the substrate. Roundup Weed & Grass Killer Super Concentrate® was non-toxic to both species when kept on soil; with paper towels this formulation was practically non-toxic to spadefoots, but killed more than half of the toads. Comparable to tadpoles, also metamorphs show great differences in sensitivity to different herbicide formulations, depending on the species studied. Soil usually lowered the toxicity: all animals survived the treatments to the herbicides when kept on soil, with the exception of Roundup Weed & Grass Killer Ready-To-Use Plus ®. This most toxic Roundup® formulation is labelled for private use and commonly applied to residential lawns and gardens, whereas both formulations used in agriculture (Ignite 280 SL® and Roundup WeatherMAX®) did not kill any individual kept on soil. Hence, DINEHART et al. (2009) concluded that the tested agricultural formulations were not likely to affect short-term survival of the two anuran species in real-world scenarios, in contrast to the formulations labelled for private use.

Ignite 280 SL® 0.21 mL glufosinate/m^2.

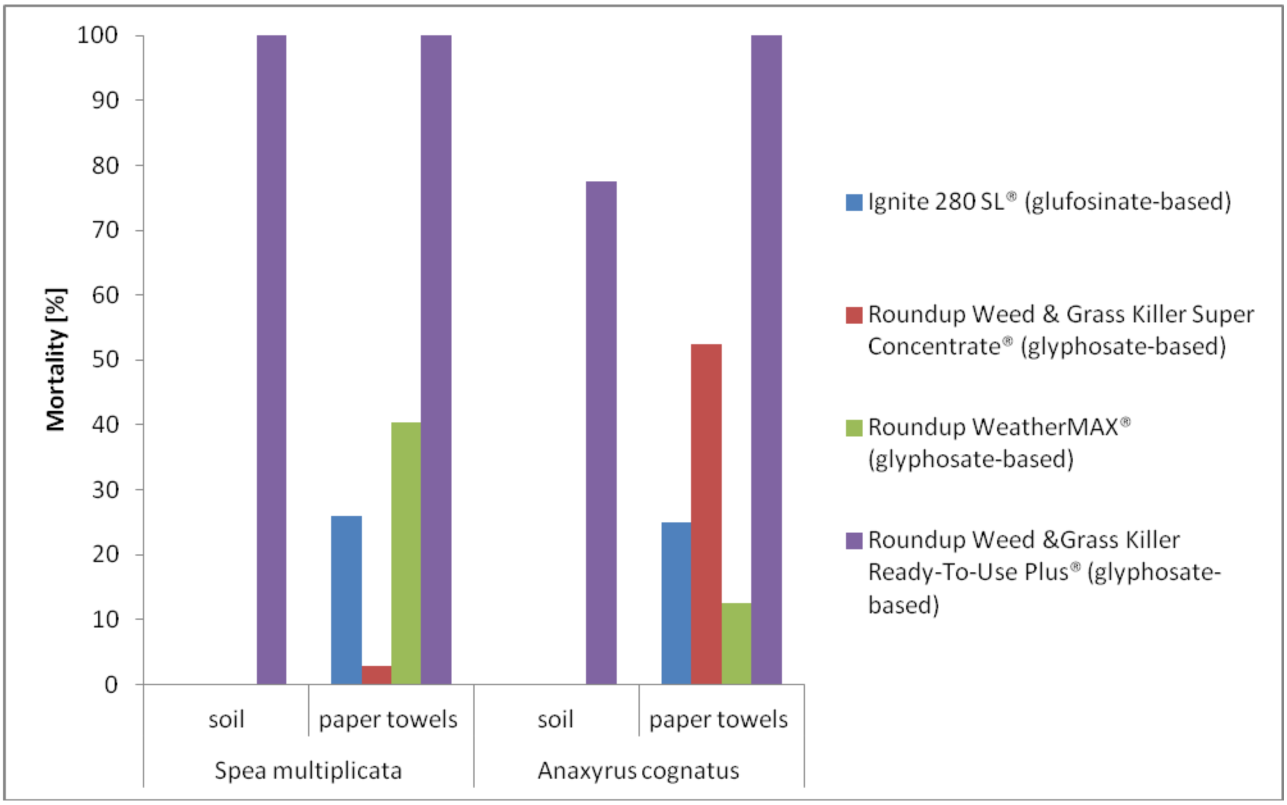

Fig. 13: Acute toxicity of different herbicides to metamorphs of two North American anurans. Animals were kept on soil or paper towels and over-sprayed at maximum recommended application rates. Soil usually increased survival, except for one GBH (data from DINEHART et al. 2009).
Note that mortality in the *Anaxyrus cognatus* control group was 2.5%, but 28.5% in the *Spea multiplicata* control group. Hence, the *S. multiplicata* results should be viewed with caution.

In a subsequent study, DINEHART et al. (2010) investigated the acute and chronic toxicity of both formerly tested agricultural formulations, Roundup WeatherMAX® (GLY-based) and Ignite 280 SL® (glufosinate-based) to larval New Mexico spadefoots (*Spea multiplicata*) and Plains spadefoots (*S. bombifrons*). Larvae of both species were field-collected from three cropland sites and two grassland sites, i.e. with marginal surrounding agriculture[61]. In the first experiment of this study, larvae in Gosner stage 29 or 30 (cf. GOSNER 1960) were exposed to eight herbicide concentrations for 48h for acute toxicity tests[62] and then survivors were kept without herbicides for another week (plus 168 h) and observed for chronic effects. No mortalities occurred in the control groups and the lowest treatments of both herbicides. No significant differences between Roundup WeatherMAX® LC50 values for Plains or New Mexico spadefoots were detected (see Table 17). Likewise, no significant difference in mean body weight of both *Spea* larvae could be observed, when the same land use types were compared. The two studied species were generally more tolerant to the glufosinate-based herbicide than to the GBH (Table 17)[63]. LC50 values were similar

61 Larvae were first held and tested as a mixed species culture because protein electrophoresis is required to differentiate larvae of these species. Identification via protein electrophoresis occurred after termination of the experiment. Due to their limited availability, the highest dose of each herbicide was excluded from tests with grassland larvae.

62 Roundup WeatherMAX® 0.75, 1.5, 2.25, 3, 4.5, 6, 7.5, 10 mg glyphosate/L; Ignite 280 SL® 0.5, 2.5, 3.75, 5, 7.5, 10, 12.5, 15 mg glufosinate/L.

63 $LC50_{48\text{-}h}$ for *S. bombifrons* tadpoles from grasslands was 3.55 mg glufosinate/L, from croplands 3.70; $LC50_{48\text{-}h}$ for *S. multiplicata* larvae from grasslands was 5.55 mg glufosinate/L, from croplands 4.85.

among populations living under different land use. *Spea multiplicata* was more sensitive to Ignite 280 SL®[64]. In the second experiment, DINEHART et al. (2010) chronically exposed Gosner stage 29-30 (cf. GOSNER 1960) spadefoot tadpoles to two concentrations of both herbicides mentioned. All concentrations[65] were predicted environmentally relevant. Every four days, 80% of the water was changed and treatments were renewed. Survival was monitored for 30 days[66]. Survival of both species significantly differed among treatments, but not species or land use. No Plains spadefoot tadpole survived for 30 days of exposure to both concentrations of Roundup WeatherMAX®[67]. All New Mexico spadefoot larvae exposed to the high concentration of Roundup WeatherMAX® died within the first five days. Compared to Roundup WeatherMAX®, neither Plains nor New Mexico spadefoot survival was affected due to exposure to both concentrations of Ignite 280 SL®. DINEHART et al. (2010) concluded that exposure to the glufosinate-based herbicide at environmentally relevant concentrations does not result in high rates of mortality among tadpoles of the two spadefoot species. Acute and chronic toxicity tests indicated that the glufosinate-based herbicide is less toxic than the GBH to larvae of the tested species. Chronic exposure at environmental predicted concentrations of Roundup WeatherMAX® may cause extensive mortality among larvae of the two spadefoot species.

Hormonal effects?

In a study on the combined impact of a fertiliser and Roundup Plus® on the Gold-striped salamander (*Chioglossa lusitanica*) (compare chapter 5.6.2.1), 1 or 2 mg herbicide a.e./L neither had a significant lethal effect on embryos nor on hatching time or developmental stage. However, embryos were – unexpectedly – significantly larger at hatching than controls (ORTIZ-SANTALIESTRA 2011). One could postulate that the body is enlarged because the herbicide impacts the thyroid hormonal balance as observed in other studies (e.g. GUTLEB et al. 2000), but this was not investigated in the study by ORTIZ-SANTALIESTRA (2011).

Comparison of a GBH with selective herbicides at environmentally relevant concentrations

WILLIAMS & SEMLITSCH (2010) exposed tadpoles of three North American anurans[68] to herbicide concentrations drawn directly from monitoring data of stream water quality (averages) and from drinking water standards. Active ingredients of tested herbicides were atrazine, S-metolachlor and GLY[69], which are among the most common pollutants found in Midwestern

64 The LC50 values were 56% and 31% higher for tadpoles of this species than for *S. bombifrons* tadpoles from both grassland and cropland sites.
65 Roundup WeatherMAX® test solutions contained 2.8 mg a.e./L or 2.0 mg a.e./L and those of Ignite 280 SL® 1.0 mg glufosinate/L or 0.5 mg glufosinate/L.
66 After being euthanized, larvae were identified as New Mexico or Plains spadefoot by protein electrophoresis.
67 All larvae exposed to 2.8 mg a.e./L died within the first two days, while some larvae exposed to 2.0 mg a.e./L survived 11 days.
68 *Anaxyrus americanus, Pseudacris triseriata* and *Hyla versicolor.*
69 In this study, atrazine was added as Atrazine 4L® and S-metolachlor as Dual II Magnum®. Two

streams. Tadpoles at Gosner stage 25 (cf. GOSNER 1960) were assigned to a single herbicide treatment[70]. Water was changed and treatments renewed every three days. Study time was equal to the larval period of each species. Metamorphosis was defined as emergence of at least one forelimb (i.e. stage 42, according to GOSNER 1960). Survival, mass at metamorphosis and length of the larval period were considered. When *Pseudacris triseriata* tadpoles were exposed to the highest Roundup WeatherMAX® concentration (i.e. only to 572 ppb a.e. ≈ 0.572 mg a.e./L) only 20% survived compared to 70-90% for all other treatments. Survival of *Anaxyrus americanus* and *Hyla versicolor* tadpoles was unaffected by all treatments. However, in case of *A. americanus* but not *H. versicolor,* the larval period was significantly lengthened by two treatments: Tadpoles of this toad metamorphosed 14% later when exposed to the high Roundup WeatherMAX® concentration and 8% later when exposed to the high Roundup Original Max® concentration. The high Roundup WeatherMAX® concentration also lengthened metamorphosis of *P. triseriata* tadpoles by 13%[71]. Mass at metamorphosis did not differ with herbicide treatment for any of the three species. The results of WILLIAMS & SEMLITSCH (2010) are important in different respects. (i) They indicate that environmentally relevant concentrations of Roundup WeatherMAX®, as low as national drinking water standards, kill high proportions (80%) of the *P. triseriata* tadpoles. (ii) Mortality appears to be associated with the surfactant present in this GBH. These results highlight the formulation and species-specific sensitivity, as already stated by previous authors. (iii) The larval stage period of *P. triseriata* and *A. americanus* was lengthened by Roundup WeatherMAX® and Roundup Original Max® lengthened the larval stage period of the toad tadpoles. These metamorphic delays are in accordance with the results of HOWE et al. (2004), but CAUBLE & WAGNER (2005) found accelerated metamorphosis due to treatment by GBH. Thus, different species may show different effects to particular GBH with regard to the time to metamorphosis. A delayed metamorphosis is of unknown biological importance, but could have disproportionate consequences to amphibian species such as *A. americanus* that often oviposits in ephemeral ponds. More tadpoles could die when the ponds are drying out in the summer because metamorphosis is not completed.

Again GBH and surfactants

LAJMANOVICH et al. (2011) determined different LC50 values (see Table 17) of Gosner stage 36-38 tadpoles (according to GOSNER 1960) of the toad *Rhinella arenarum*. Significantly different toxic effects of different formulations have been found, as in previous studies, too. The main finding of the study was that the tested GBH are inhibitors of enzymes taking part in metabolism and detoxification (for details on the study see chapter 5.5.1).

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[70] glyphosate formulations were tested, Roundup WeatherMAX® and Roundup Original Max®. Atrazine 0.2 or 3 ppb a.i., S-metolachlor 0.2 or 10 ppb a.i., glyphosate 0.6 ppb or 700 ppb a.i. (for each of the two formulations).

[71] This result should be viewed with caution because only two *P. triseriata* tadpoles survived this herbicide treatment.

FUENTES et al. (2011) compared the acute toxicity of Roundup Original® and Roundup WeatherMAX® to six North American anuran larvae[72]. Based on the calculated $LC50_{96\text{-}h}$ values (non-renewal; see Table 17), four species were more sensitive towards Roundup WeatherMAX® and two species towards Roundup Original®. Two of six species (*Anaxyrus fowleri*, *Lithobates clamitans*) responded significantly different to the two formulations tested. The authors supposed that the increased sensitivity to Roundup WeatherMAX® compared to Roundup Original® was likely due to the kind or relative amount of surfactant included. This study shows – one more time – the formulation and species-specific sensitivity of tadpoles.

5.4.2 Mesocosm studies

RELYEA (2005c) examined the impact of four globally common pesticides on aquatic organisms, which were the insecticides carbaryl and malathion and the herbicides 2,4-D and a Roundup® formulation. He used a completely randomised design and simulated semi-natural aquatic communities in mesocosms (including zooplankton and phytoplankton, water snails as food competitors, larval salamanders (*Ambystoma maculatum*) and ten different predatory water insects [larvae or imagines]). Tadpoles of five anuran species[73] were added to each tank and pesticides were applied at the maximum application rate[74]. After two weeks, the experiment was terminated and it was found that most pesticides significantly reduced species' richness. Pesticides had a significant effect on survival except for *Anaxyrus americanus* and *Pseudacris crucifer* tadpoles. While *Lithobates pipiens* and *Hyla versicolor* tadpoles were completely eliminated by Roundup®, *Lithobates sylvaticus* survival was reduced only by 2%. The observed high tadpole mortality was due to direct toxicity of the pesticides and not due to destruction of the algal food source, as much of the mortality occurred within the first day, and Roundup® actually increased the biomass of periphyton (i.e. the food source) by 40%. Biomass of tadpoles and snails significantly decreased with Roundup®. Richness of tadpoles and snails was only reduced by Roundup® and predator biomass was significantly lower with the insecticides and Roundup®. While 2,4-D had no effect, general species richness was 15% lower with carbaryl, 30% lower with malathion and 22% lower with Roundup®. As supposed, richness of predatory insects and zooplankton was significantly reduced by the insecticides. Survival of tadpoles increased with addition of insecticides because concentrations well under LC50 values for the tested amphibians were used and because of the high predator mortality. All pesticides – with exception of 2,4-D – had further indirect impacts on the aquatic community. The results imply that different pesticides can have a profound impact on the diversity and productivity of aquatic communities within only two weeks. Thus, in assessing the impacts of insecticides on amphibians, it is critical to consider the ecological context. Nevertheless,

[72] *Lithobates catesbeianus, L. clamitans, L. pipiens, L. sphenocephalus, Anaxyrus fowleri* and *Hyla chrysoscelis.*

[73] *Anaxyrus americanus, Hyla versicolor, Lithobates sylvaticus, L. pipiens* and *Pseudacris crucifer.*

[74] As recommended by the manufacturer. For the Roundup® formulation, this was 3.8 mg a.i./L ≈ 2.85 mg a.e./L.

all in all, Roundup® had the largest effect on the amphibian community. It reduced tadpole richness by 70%, entirely killed two species and nearly eliminated a third – while it had no effect on three other species, however[75].

In probably the best-known study on the impact of GBH on amphibians, RELYEA (2005b) applied 3.8 mg a.i./L (≈ 2.85 mg a.e./L) of Roundup Weed & Grass Killer® to outdoor pond mesocosms (with no soil, with sand or with loam). After a periphyton community was established in the pond mesocosms as food source, tadpoles at Gosner stage 25 (cf. GOSNER 1960) of three naturally co-existing North American amphibian species were added[76] followed by herbicide treatment two days after. The experiment was terminated 20 days after herbicide treatments because the toads of the no-herbicide treatments were approaching metamorphosis. The GBH caused a highly significant reduction in survival of all three species. Across all three soil experiments, the applied concentration of this GBH reduced survival of *Hyla versicolor* from 75% to 2%, of *Anaxyrus americanus* from 97% to 0% and of *Lithobates pipiens* from 98% to 4% (see Fig. 14). Taken together, only 2% of all tadpoles survived the herbicide treatment after 20 days. RELYEA (2005b) stated that death appeared to result from direct toxicity rather than from any indirect effect of the herbicide in the mesocosms (i.e. reduction of the algal food resource) because (i) numerous dead larvae were observed within the first day; (ii) in another mesocosm experiment, a GBH rather increased than decreased periphyton biomass (see RELYEA 2005c); (iii) in a laboratory study (RELYEA 2005a) – where tadpoles were fed ground fish flakes – the GBH also caused rapid death. Furthermore, the results of this study suggest that Roundup® killed amphibian larvae before it was adsorbed to soil or sediment because the addition of loam or sand did not reduce its toxicity. Instead, loam significantly reduced survival in *H. versicolor* compared to no soil or sand treatments. These findings are contrary to those of BERNAL et al. (2009b) (see 5.4.1.).

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75 It is difficult to assess the effect because only few toads survived the control.
76 *Anaxyrus americanus, Hyla versicolor, Lithobates pipiens.*

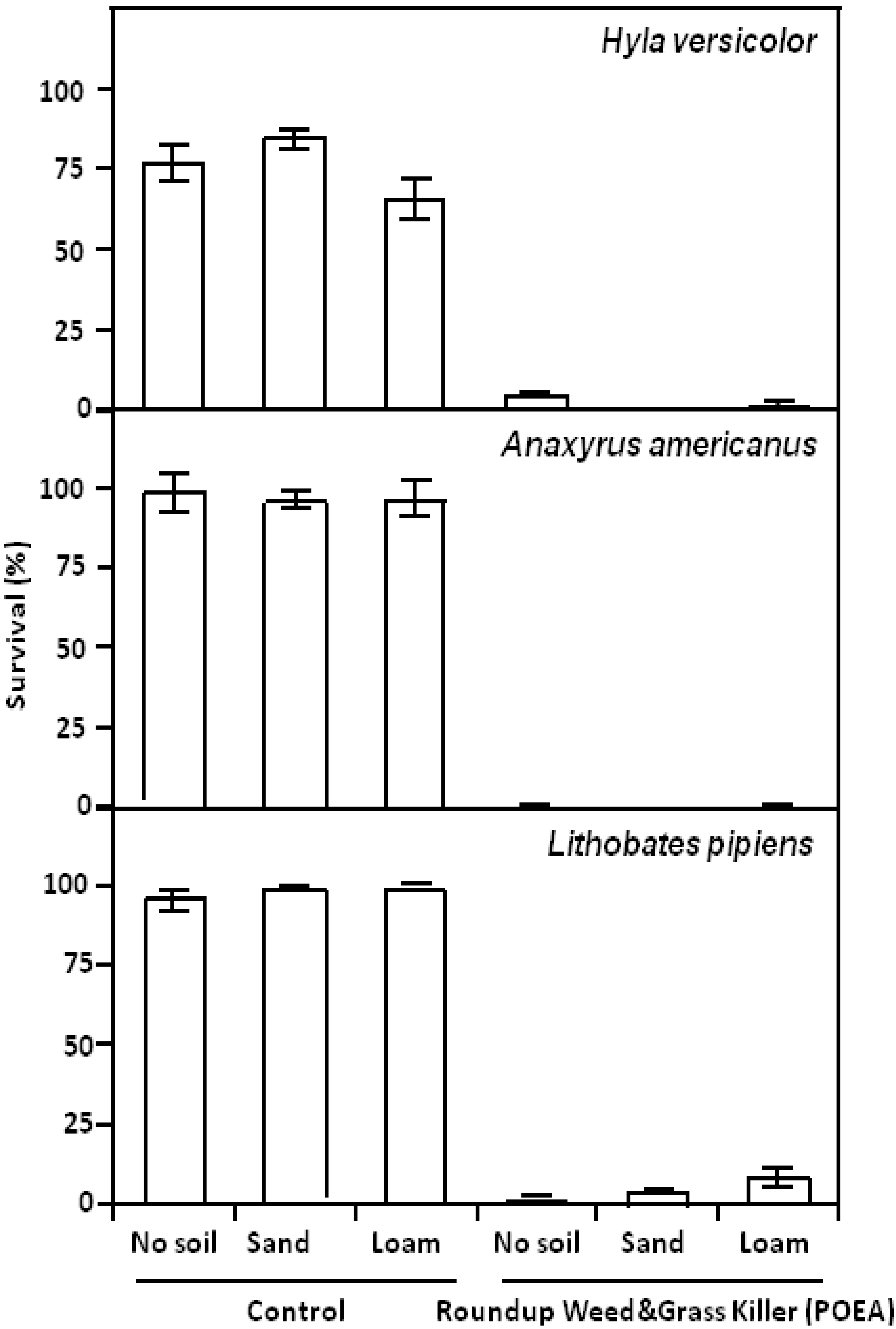

Fig. 14: Acute toxic effects of a single application of a GBH on survival of tadpoles of three North American species: Soil did not reduce adverse effects
(drawn after RELYEA 2005b).

Besides the aquatic experiment, RELYEA (2005b) also studied the effect of direct over-spraying in a worst-case scenario (i.e. no interception by vegetation). Post-metamorphic treatment with 6.5 mL of Roundup Weed & Grass Killer® killed on average 79% animals of all three tested species within 24 hours: Survival of *H. versicolor* juveniles was reduced from 100% to 18%, that of *L. sylvaticus* juveniles from 96% to 32% and that of *A. woodhousii* from 100% to 14%. In conclusion, amphibian juveniles are considered to be highly sensitive to direct over-spraying with this GBH at the tested application rate.

In another mesocosm study by RELYEA (2009) on the effect of pesticide mixtures, GLY alone at a much lower concentration (10 ppb) did not affect tadpole survival, mass at metamorphosis or time to metamorphosis (for details on this study, see chapter 5.6.1.1).

In an outdoor mesocosm experiment[77], JONES et al. (2010) studied the impact of the exact exposure time and of multiple small doses versus a single large dose. Two species of anurans (*Anaxyrus americanus*, *Lithobates sylvaticus*) were exposed to 11 treatments of Roundup Original Max® in a randomised design. Three concentrations (1, 2 and 3 mg a.e./L) were applied once on either day 0, 7 or 14 of the experiment. Furthermore, two concentrations (0.33 and 1 mg a.e./L) applied repeatedly at three time points (on days 0, 7 and 14) simulated an event in which a pond is contaminated on three occasions within three weeks, a scenario that could happen, for instance, due to three heavy rainfalls. *L. sylvaticus* tadpoles at Gosner stage 26 and *A. americanus* tadpoles at Gosner stage 25 (cf. GOSNER 1960) were tested together. Periphyton biomass increased over time across all treatments. The experiment was terminated on day 18, because toads were about to metamorphose. There was a significant multivariate effect of all treatments on amphibian survival and mass. Survival of *L. sylvaticus* tadpoles was significantly reduced with early and midway applications of 2 and 3 mg a.e./L and late applications of 3 mg a.e./L. For *A. americanus* tadpoles, survival was significantly reduced with early application of 2 and 3 mg a.e./L and midway and late application of 3 mg a.e./L. Hence, the impact of Roundup Original Max® on the tadpoles varied with both application time and concentration. For both species, LC50 values for early and midway applications were similar, but significantly lower than LC50 values for late applications[78]. The mass of *L. sylvaticus* was significantly reduced with late application of 3 mg a.e./L, whereas that of *A. americanus* was significantly reduced with midway and late applications of 2 and 3 mg a.e./L. Thus, the impact on mass also varied with both application time and concentration. In comparing three applications of 0.33 mg a.e./L to one application of 1 mg a.e./L, no differences in survival or mass of both species could be observed. However, in comparing three applications of 1 mg a.e./L to one application of 3 mg a.e./L, the single application caused lower survival in both species, independent of application time. This is probably because single applications had higher peak concentrations compared to the three smaller applications. Furthermore, the single 3 mg

[77] The mesocosms were 750-L cattle tanks filled with about 580 L of water. For periphyton growth, initially rabbit chow and oak leaf litter were added to each tank. In addition, two aliquots of local pond water containing zooplankton, phytoplankton and periphyton were added.

a.e./L application significantly reduced the mass of both species, but only when it was applied later in the experiment. For *L. sylvaticus*, increasing the treatment from 1 to 3 mg a.e./L caused a 91% decline in survival when applied early, a 82% decline when applied midway and only a 27% decline when applied late. In addition, a 27% decline in mass could be observed when applied later. For *A. americanus*, increasing the concentration from 1 to 3 mg a.e./L caused a 75% decline in survival when applied early, a 91% decline when applied midway and a 31% decline when applied late. It also caused a 16% decline in mass when applied midway and a 35% decline when applied late. In conclusion, multiple smaller applications caused weaker effects than single applications of the same total amount, even though the multiple, smaller doses showed little evidence of breakdown over time. The most striking finding was that the impact of a single application declined when applied later. Either tadpoles could be more sensitive early in their development (ontogeny) or the results may reflect an acclimation to the herbicide over time. Although the experimental design could not distinguish between these two alternatives, RELYEA (2005a) and RELYEA & JONES (2009) as well as others have proposed that the vast majority of tadpoles are killed within the first three days. The observed mass reductions were caused probably not because less food was available, but because the herbicide altered the tadpoles' behaviour or physiology. Another main finding of this study was that in the temperature-stratified water column herbicide concentrations were considerably higher near the surface than near the bottom of the tanks. This is a crucial finding because ectothermal amphibian larvae typically prefer the warmer temperatures of surface waters (at least if not shared by present predators), so in natural ponds they are likely to be exposed to higher than average herbicide concentrations.

5.4.3 Field studies

GLASER (1998) studied the effects of the GBH Vision® on the North American Wood frog, *Lithobates sylvaticus*. She sampled egg masses from ponds at different sites of pine (*Pinus banksiana*) plantations in northern Ontario (Canada)[79]. Development and growth of frogs were observed under laboratory conditions. Eggs from Vision® treated areas had smaller post-hatch size, smaller juvenile lengths and masses and more post-hatch deformities. GLASER (1998) concluded that the use of Vision® negatively affected the reproductive success of Wood frogs on treated sites. Possible effects of Vision® were most likely maternal (and should not be carried over into the next generation). The author concluded only negligible effects on populations because Vision® was rarely sprayed in the given plantations, but in areas with repeated spray of the herbicide (for example in agricultural crops) effects could be substantial.

THOMPSON et al. (2004) conducted field experiments on the potential effects of Vision® spraying in northern Ontario forest plantations. Over a period of three applications (1999-2001),

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[78] For LC10, LC50 and LC90 values see JONES et al. (2010: 2020).

[79] These were control sites, mechanically disturbed sites, sites where Vision® was sprayed once with 1.44 kg a.e./ha, sites where Vision® was sprayed once with 1.8 kg a.e./ha and sites where Vision® was sprayed once with each 1.44 and 1.8 kg a.e./ha.

tadpoles of *Lithobates clamitans* and *L. pipiens* were first sampled in undisturbed areas and then transferred to ponds and caged. Ponds were over-sprayed (at 1.07-2.14 kg a.e./ha, overall average of 1.92 kg a.e./ha) and had either no, adjacent or vegetated buffers (buffers ranged in size from 30 to 60 m). Vegetated buffers significantly reduced exposure. Mean concentrations were about 0.03 mg a.e./L in buffered wetlands, about 0.18 mg a.e./L in adjacent wetlands and about 0.33 mg a.e./L in wetlands without buffers, but values ranged from undetectable (<0.01 mg a.e./L) to quite high (1.95 mg a.e./L). Mean tadpole mortality ($LC50_{48\text{-}h}$) was similar for both species at Gosner stage 25 (cf. GOSNER 1960). Although differences in mean mortality for *L. clamitans* were observed – with the highest mortality in over-sprayed wetlands – THOMPSON et al. (2004) concluded that typical exposure in forest wetlands is insufficient to induce acute mortality in these Canadian amphibians because the differences were not statistically significant. The main weak point in this study (THOMPSON et al. 2004) is that the authors used the buffered wetlands, which contained almost no herbicide, as control for their calculations, but 15% of *L. pipiens* larvae and 26% of *L. clamitans* larvae died in these 'controls'. A higher survival in this 'control group' might have contributed to statistical significance. It is a good standard in toxicological studies that no more than 10% of control animals should die. Since the study does not meet this standard, for instance, RELYEA (2011) disputes the calculated non-significant differences in survival and the conclusion that aerial applications of Vision® pose low risk to amphibians.

Pesticide avoidance

TAKAHASHI (2007) investigated the impact of both predatory stress and water contamination with GBH on oviposition site selection. He observed that Gray treefrogs, *Hyla versicolor*, completely avoided breeding when a GBH together with predators (fish) or even just the herbicide was present, but at a relatively high concentration of 2.4 mg a.e./L and only five pairs contributed to the results and spawned in the controls or pools with fish. Furthermore, VONESH & KRAUS (2009) supposed that pesticide avoidance is species-specific because treefrogs but not Northern cricket frogs significantly avoided artificial ponds, which were contaminated with an insecticide. It seems relevant to test the impact of low GLY concentrations (that commonly can be found in the environment) on site selection of amphibians. Findings of WAGNER & LÖTTERS (2013) suppose that neither adult Common frogs, Palmate newts (*Lissotriton helveticus*) nor Alpine newts (*Ichthyosaura alpestris*) are able to perceive environmentally relevant concentrations of AMPA (0.005-0.5 µg/L), GLY and Roundup LB-PLUS® (0.01-1 mg a.e./L, respectively) in artificial pools. Hence, a general avoidance of water bodies, which were contaminated with GBH remains unclear (see also chapter 5.6.1.2).

5.4.4 Interim conclusion and discussion for chapter 5.4

Thirty-three studies published between 1997 and 2011 were retrieved and analysed which investigated effects of GLY, its formulations and surfactants as a single stressor in the laboratory, mesocosms and the field. The majority of studies (about 85%) looked for acute toxic effects, and there is consistency, that GBH affect survival of amphibians in a dose-response manner.

Twenty-two studies tested tadpoles, eight anuran juveniles or adults; only four tested anuran embryos, only one larval urodels and only one embryonic urodels. Therefore, most findings are restricted to tadpoles. Around 50 different amphibian species were tested in the retrieved studies, but there are of course preferred organisms such as *Lithobates pipiens, L. clamitans* or *Xenopus laevis*. However, study designs varied widely and parameters such as renewal of substances, study time, tadpole density or pH value are important and might explain why differing results are reported sometimes.

With regard to GBH and their surfactants, LC50 values ranged from 0.2 to over several hundred mg a.e./L for tadpoles and embryos of different species and formulations. Embryos are more resistant than tadpoles. This is mainly due to lack of external gills where especially surfactants accumulate during filtrating. Because most LC50 values range from 1 to 10 mg a.e./L (Tables 17 and 18), **most GBH can be seen as 'moderately toxic' (1 mg/L<LC50<10 mg/L) for aquatic life-stages of anurans**. However, **some formulations are 'highly toxic' (0.1 mg/L<LC50<1 mg/L) for tadpoles of some anuran species**. Furthermore, terrestrial life-stages are more resistant than aquatic life-stages (cf. Tables 16-18 with 19).Hence, **acute toxicity of GBH and their surfactants is species-, life-stage- and formulation-specific**. The degree of acute risk depends on the amphibian species and especially on the formulation because the a.i. alone (i.e. GLY) has much less acute toxic effects than its various formulations (cf. Tables 16 and 19 with Tables 17 and 18). LC50 values of >17.9 or >400 mg a.e./L mean that at this highest concentration in the laboratory test no effect was observed. Hence, **GLY acid and isopropylamine salt are 'slightly toxic' (10 mg/L<LC50<100 mg/L) to 'practically non-toxic' (LC50>100 mg/L) to aquatic and terrestrial life-stages of anurans**. The lowest LC50 value for the a.i. (6.5 mg/L) was calculated and in the laboratory study, tadpoles were exposed to Rodeo® with a surfactant. Therefore, this result should be viewed with caution.

Many authors conclude that the added substances, namely the surfactants, are responsible for the higher toxicity of GBH. **There is hardly any dispute about the relevance of the surfactants**. Studies published at the beginning, mid and end of the considered period support this view. POEA is especially mentioned several times, but also formulations with unknown surfactants can be 'highly toxic' to tadpoles (see results from RELYEA & JONES 2009; Table 17). Published LC50 values from experiments using **pure surfactants** (POEA, NPE and alcohol alkoxylate) range from 1.7 to 9.2 mg/L **for aquatic life-stages of anurans ('moderately toxic': 1 mg/L<LC50<10 mg/L**; Table 18**)**. Higher toxicity of some formulations may be due to species-specific sensitivity or differences in the laboratories.

When maximum concentrations of the a.i., especially the estimated worst-case EECs (0.9 to 7.6 mg a.e./L; see chapter 5.2) are achieved in natural ponds, it is noteworthy that several formulations and their surfactants, respectively, would kill high proportions of tadpoles (based on LC50 values) while other formulations would not so (see Table 17). It has to be noted again that measured and estimated GLY concentrations can only be seen as raw approximations for amphibians' risk when the applied formulation and its composition – i.e. its surfactant system and adjuvant – are unknown. Therefore, **wild amphibian's risk assessment has to be conducted at a local scale** by considering important variables (e.g. applied formulation, species and life-stage present, application method, kind of water body, presence of buffer strips).

Effects and delayed effects of singly applied or chronic exposure to sublethal doses were considered by only nine of the retrieved studies. It is an issue of more recent studies and related topic here is, for instance, endocrine disruption. So far, effects of GBH on tadpoles were investigated. Considered endpoints were mainly growth (size and mass at metamorphosis) and time to metamorphosis. Sublethal concentrations of some GBH apparently have the potential to (i) disrupt larval development, which can lead to either precipitated or delayed time to metamorphosis and mainly decreased size and mass; (ii) cause abnormal gonads (but observed in only one study); (iii) inhibit specific enzymes, which indicates general stress and low levels of individual fitness; (iv) be genotoxic and mutagenic but with unknown effects on individuals and their offspring. An evaluation of long-term effects is difficult because specific life cycle tests with amphibians have never been conducted yet (in already conducted tests, specimens are anesthetized after metamorphosis). With regard to amphibians in the wild, smaller individuals have lower survival chance, for instance, due to lower over-winter survival or higher predation risk. Later metamorphosis poses risk for species that reproduce in ephemeral ponds, which dry out. Furthermore, later metamorphosis enhances the risk of predation by aquatic predators (invertebrates, fish etc.). Abnormal gonads may have influence on the reproduction community.

There are many other variables that have to be considered when results from laboratory studies are transferred to the real-world. Thus, the interactions with other stressors are part of an own chapter (5.6).

Concerning the results of the conducted laboratory studies with GLY or GBH as a single stressor, we conclude that more standardized laboratory experiments on chronic effects and orally administered formulations (not only the a.i. GLY) to juveniles and adults are necessary for further conclusions. With regard to dermal uptake of substances by terrestrial life-stages amphibians, a standard method shall be introduced to official risk assessment (cf. method by QUARANTA et al. 2009).

5.5 How do glyphosate and its formulations affect amphibian health?

Main questions remain how GLY and GBH affect amphibian health. In the following, known reasons for acute toxicity and delayed effects after chronic or short-term exposure to sublethal concentrations are summarised.

5.5.1 Phenotypic effects and reasons for acute toxicity

Lethal effects of GLY and GBH on tadpoles were caused by damages of the gills. Embryos are therefore supposed to be less susceptible due to lack of fully developed gills. By studying effects of treatment with herbicides on detoxificating enzymes, oxidative stress was observed that could be another mechanism of toxicity.

LAJMANOVICH et al. (2003) found larval malformations in Neotropical treefrog tadpoles (*Scinax nasicus*). Malformations increased with time and herbicide concentration. The gills in particular were very sensitive, and likewise to different herbicides (LAJMANOVICH et al. 1998). Hyobranchial skeletons showed alterations in their cartilage structure, i.e. collagen was disrupted. In extreme cases, branchial arches were partially destroyed. External malformations of tadpoles amounted to cranial and mouth deformities, eye abnormalities and bent curved tails. LAJMANOVICH et al. (2003) suspected the surfactant POEA to be responsible for those malformations.

In addition, HOWE et al. (2004) named POEA as principally responsible for observed tail damages and acute toxicity. Surfactants interfere with gill morphology in fish and cause lysis of gill epithal cells (PARTEARROYO et al. 1991). Therefore, HOWE et al. (2004) assumed the same cause for acute toxicity to amphibian larvae as in fish. Embryos could be more resistant than tadpoles due to the lack or insensitivity of target organs, such as functional gills.

Malformations of embryos occurred for both *Lithobates pipiens* (mainly lateral bent tails) and *L. clamitans* (abnormal face, eye and gut development) in the experiment by EDGINTON et al. (2004). They supposed that gills take up POEA readily and accumulate it best when POEA is present in the non-ionised form. The ionised form of POEA is predominant at pH 6.0 and the non-ionised form at pH 7.5. They proposed that this circumstance may be responsible for the observed higher mortality at higher pH levels.

LAJMANOVICH et al. (2011) studied which enzymes were inhibited by certain GBH. Several other authors already studied enzymes involved in metabolisation and detoxification of agrochemicals as biomarkers in order to assess potential risks of pesticides on amphibians. For example, B-type esterases are good candidate enzymes. Another one is the group of glutathione S-transferases which are commonly used as a biomarker of many contaminants (see citations in LAJMANOVICH et al. 2011). For the study, Gosner stage 36-38 (cf. GOSNER 1960) tadpoles of *Rhinella arenarum* that are commonly found in Neotropical agricultural areas were field-collected. Seven tadpoles per tank were used in the experiments and treatments were triplicate with chemicals ranging from 1.85 to 240 mg a.e./L to determine acute toxicity values (cf. chapter 5.4.1). Animals with a survival rate > 85% were euthanized and used to measure enzymatic activities. B-esterases and glutathione S-transferases activities significantly varied between different formulations and concentrations of GBH. It remains unclear whether GLY or POEA were responsible for the observed effects. Depletion of glutathione S-transferases results in oxidative stress. Hence, environmental relevant concentrations (survival rate >85%) of GBH potentially induce oxidative stress. This is a novel insight in the mechanism of toxicity of GBH.

5.5.2 Immunosuppressive effects

Amphibians have effective, diverse immune systems, but recent outbreaks of severe diseases (like chytridiomycosis, see chap. 5.6) occurred that led to population declines and extinctions. CAREY et al (1999) suggest that the responsible disease agents have emerged only recently and/or that anthropogenic co-stressors compromise the response of the immune system.

CAREY et al. (1999) summarised the patterns in pathogen-related amphibian declines, i.e. due to emerging infectious diseases (see also chapter 5.6.1.1). They further discussed general hypotheses concerning the apparent failure of amphibian immune systems to resist disease agents. Six hypotheses were named: (i) the new pathogen is highly virulent and kills an amphibian before its immune systems can be mobilised, (ii) the pathogen is immunosuppressive, (iii) habitat changes and/or contamination alter the microflora in the soil and in the water, so that a previously rare pathogen becomes prevalent, (iv) environmental changes stress amphibians, so that they produce stress hormones, which increase the growth rates (i.e. virulence) of pathogens, (v) sublethal environmental changes cause neuroendocrine changes reflecting stress, which result in immunosuppression and (vi) exposure to contaminants directly diminishes components of the immune system; immunosuppression, in turn, results in increased vulnerability to diseases and/or opportunity for pathogens to switch hosts. The latter two supposed reasons can be caused by pesticide applications. Many chemical contaminants, such as herbicides, are already tested for acute toxicity to amphibian embryos (cf. DEVILLERS & EXTRAYAT 1992), but there exist few studies on the immunosuppressive behaviour of those pesticides (but see KIESECKER et al. 2002; CHRISTIN et al. 2003; GENDRON et al. 2003; BRODKIN et al. 2007 for studies showing immunosuppression of atrazine in amphibians).

HAYES et al. (2006) exposed larval *Lithobates pipiens* and adult *Xenopus laevis* to low concentration (0.1 ppb each, 0.1 or 10 ppb mixtures) pesticide mixtures to study sublethal effects (cf. chapter 5.5.3). GBH were not included in the study. Because animals exposed to a nine-pesticide mixture suffered from increased disease rates (flavobacterial meningitis), they examined the thymus of the larval frogs as a measure of immune function. Exposure to S-metolachlor and atrazine, two common herbicides, damaged the thymus as measured by increased thymic plaques. The frequency of damage further increased when animals were treated with pesticide mixtures. Effects on plasma corticosterone levels in adult *X. laevis* were examined using hormone radioimmunoassay (for details of methods see HAYES et al. 2006). Corticosterone can cause all the negative effects observed with pesticides including retarded growth, development and immunosuppression (see citations in HAYES et al. 2006). They found that the pesticide mixtures had a significant effect on corticosterone levels in *X. laevis*. Levels increased fourfold. HAYES et al. (2006) found some more negative effects in their experiment (effects on the hormone system, i.e. on sexual differentiation, and decreased growth), but they stated that “the immunosuppressive effects are likely more relevant” (HAYES et al. 2006: p. 48). A related study specific for trematode infections caused by GLY, is mentioned in ROHR et al. (2008) in chapter 5.6.1.1; for chytrid infections in GAHL et al. (2011) in the same chapter.

5.5.3 Endocrine disruption

HOWE et al. (2004) observed that TRβ mRNA expression significantly increased in young tadpoles of anuran larvae (see Table 17). Tail regression during metamorphosis requires thyroid hormone-dependent changes in gene expression and the genetic program is regulated by thyroid hormone-specific nuclear transcription factors (SHI 2000). One of these factors, TRβ, can be used to detect perturbation in thyroid hormone signalling. To determine whether the incidence of tail damage was associated with altered TRβ mRNA expression, levels of TRβ mRNA were assessed in tail samples of Gosner stage 25 and 42 tadpoles (cf. GOSNER 1960) after GLY exposure. In Gosner stage 25, tadpoles exposed to the high concentration of Roundup Original® and high and low concentrations of Roundup Transorb®, a significant increase of TRβ mRNA could be detected. Conversely, Gosner stage 42 tadpoles showed no significant differences compared to the control. However, the observed tail reduction and the presence of abnormal gonads in tadpoles are sufficient to suppose that POEA and both Roundup formulations have disrupted the endocrine system.

Alterations of TRβ mRNA expression can lead to precipitated (e.g. CAUBLE & WAGNER 2005) or delayed time to metamorphosis (e.g. HOWE et al. 2004; WILLIMAS & SEMLITSCH 2010). Precipitated metamorphosis comes along with decreased size at metamorphosis, i.e. reduced fitness of metamorphs. Delayed metamorphosis of tadpoles living in ephemeral ponds can lead to a higher mortality rate as ponds may dry out. For example, a strategy for the life history of the Natterjack toad (*Epidalea calamita*) – a European ‘strictly protected’ species also occurring in Germany – is a short time to metamorphosis (within 1-2 months). Thereby, the toad can use flat ephemeral ponds with little predation pressure for reproduction. In former times, these ephemeral water bodies were mainly situated in natural floodplains, today the toad mainly uses secondary habitats like sand pits, but also puddles on agricultural lands.

There are no particular studies on the impact of GBH on amphibian sex hormone balance. HOWE et al. (2004) observed gonadal abnormalities, but could not show significant differences in sex ratios. However, SOSO et al. (2007) showed that Roundup WG® applied at 3.6 mg a.e./L negatively affected reproductive success of the fish species *Rhamdia quelen* by altering the steroid profile of females. Forty days after consecutive treatments, levels of the most important ovarian steroid, 17β-estradiol, were significantly reduced due to herbicide treatment. A lower number of viable swim-up fry were obtained in the herbicide treated group, suggesting that GBH may affect fertilisation rates, egg viability, hatching rates, embryo survival or development. Furthermore, despite a lack of statistical difference, it seems that GBH affect liver metabolism in this fish species which, in turn, might have subsequent effects. Concerning amphibians, HAYES et al. (2003) showed demasculinisation and feminisation in *Lithobates pipiens* due to exposure to 0.01 ppb of atrazine, a common herbicide.

5.5.4 Teratogenic effects

PERKINS et al. (2000) used the standardised FETAX assay to study – beside mortality – malformation rates and growth of embryos exposed to Roundup Original® and Rodeo® (see chapter 5.4.1 for detailed information). Significant increases in the incidence of malformations were not observed at any concentration that was not also lethal to the embryos at 96h. Embryo growth was not reduced at concentrations below the 96h-LC50. Using the worst-case scenario EEC of 2.8 mg a.e./L, the authors concluded a margin of safety (LC5/EEC) of about 1,900 for Rodeo® and 2.7 for Roundup Original®[80]. Hence, the authors concluded that these two GBH are non-teratogenic (but discussed the possibility that early larval stages are more susceptible due to the presence of gills and a higher surface-to-volume ratio).

Because of reports on birth defects in Argentinean regions where HR crops are widely cultivated and where GBH are extensively used, PAGANELLI et al. (2010) evaluated the potential effects of Roundup Original® on embryos of two vertebrates, namely *Xenopus laevis* and chicken. Test substances were directly injected into embryos at 1/5000 dilutions (equalling 72 mg a.e./L, i.e. nearly the eightfold LC50 found in PERKINS et al.). Embryos were incubated and later subjected to in situ hybridization, immunofluorescence and cartilage staining to visualize possible effects at molecular, biochemical and anatomical level. Main results of the study were that neural crest markers, rhombomeric patterning and primary neuron differentiation in 2-cell staged frog embryos were altered by both GLY and its formulation. Furthermore, both GLY and the formulation produced head defects and impaired the expression of dorsal midline and cephalic markers. PAGANELLI et al. (2010) investigated whether GLY and its formulation alter the expression of genes involved in head development because craniofacial defects were observed in humans living in areas with chronic exposure to GLY. They found that especially the expression of proteins that control multiple development processes was dramatically reduced at the neurula stage. The development of the craniofacial skeleton was also altered by both treatments. Eye development in particular was negatively affected as eyes disappeared or embryos exhibited cyclopia. Similar results were found in treated chicken embryos. PAGANELLI et al. (2010) suggest that GLY itself is responsible for the malformations. Because endogenous retinoic acid activity was increased in treated frog embryos and cotreatment with a retinoic acid antagonist rescued the teratogenic effects, they supposed that GLY caused the observed phenotypes by impairing the retinoic acid signaling. PAGANELLI et al. (2010) stated that the malformations observed in their study are compatible with those observed in the offspring of women chronically exposed to GBH during pregnancy. This study was followed by strong replies about the pertinence of study designs (especially high doses and injection) and conflicting interests (BVL 2010c).

5.5.5 Genotoxicity

The first study on the genotoxicity of Roundup® was performed by CLEMENTS et al. (1997). These authors exposed American bullfrog (*Lithobates catesbeianus*) tadpoles to four concentrations, 1.69, 6.75, 27 and 108 mg/L, each for 24 hours. All tadpoles in the highest concentration group died and were excluded from further analysis. After exposure, DNA damage in erythrocytes was measured using the standard 'comet' assay (see SINGH et al. 1988). Compared to a control group, no significant damage was observed at the low concentration, but at 6.75 and 27 mg/L a relationship emerged between dosage and damage. CLEMENTS et al. (1997) also tested four other herbicides and obtained similar results with two of them (AAtrex Nine-O® including atrazine, and Dual-960E® including metolachlor). The other two herbicides induced DNA damage without a dose-response (Sencor-500F® including metribuzin) or no significant DNA damage at all (Amsol including 2,4-D amine). CLEMENTS et al. (1997) concluded that use of some herbicides including Roundup® is capable of inducing DNA damage in tadpoles.

BOSCH et al. (2011) conducted micronucleus tests in post-metamorphic of *Odontophrynus cordobae* and *Rhinella arenarum* to determine genotoxic effects, i.e. the basal frequency of the micronucleated erythrocytes (MNE) were determined. They used 40 mg cyclophosphamide/L (normally used as drug and well know as a genotoxic substance) as positive control, a control group and compared them with Roundup Original®. Tested concentrations were 100, 200, 400 and 800 mg a.i./L and test duration were five days. Each experimental group and the control group consisted of three males and two females. Concentrations of 200, 400 and 800 mg a.i./L were lethal to *O. cordobae* whereas *R. arenarum* individuals only showed mild signs of toxicity. Furthermore, at the 100 mg a.i./L concentration, *O. cordobae* had a frequency of MNE twice as large than *R. arenarum*. Hence, *O. cordobae* was more sensitive than *R. arenarum*. In summary, Roundup Original® had genotoxic effects in a concentration response manner on both anuran species. On the one hand, tested concentrations were about ten times higher than those tested before on post-metamorphic amphibians via direct over-spray (e.g. MANN & BIDWELL 1999), but BOSCH et al. (2011) stated that concentrations recommended by the agrochemical industry were 10 to 20 times higher than those tested in their study.

5.5.6 Interim conclusion and discussion for chapter 5.5

Known reasons for acute toxicity and (delayed) effects after chronic or short-term exposure to sublethal concentrations of GBH are (i) *elevated malformation rates*, (ii) *gill damages* in tadpoles and (iii) *oxidative stress*.

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[80] Margins of safety > 1 are usually seen as secure.

Phenotypic malformations of embryos and larvae caused by GBH include cranial, eye and mouth abnormalities and further tail damages or bent curved tails. Also damages to the viscera were observed including gut and gonadal abnormalities. Conversely, embryos are probably more resistant to GBH due to a lack of functional gills. PAGANELLI et al. (2010) directly related observed malformations of embryos to human birth defects. Furthermore, they stated that not only GBH with their added substances are responsible for the observed teratogenic effects, but GLY itself. However, high doses (72 mg a.e./L) were used and GLY was directly injected. This study was followed by strong replies about the pertinence of study designs and conflicting interests (e.g. BVL 2010c; see also chapter 8.2). Hence, no final conclusion on teratogenic effects of GLY and GBH can be stated.

Gill damages – especially due to the surfactants – in tadpoles are probably mainly responsible for acute toxicity. Gill uptake of GBH occurs readily and that could explain the observed rapid mortality in many experiments. Furthermore, gill accumulation could accelerate due to high proportions of the non-ionised form of POEA that is predominant at higher pH levels. This explains the observed lower mortality rate in water with lower pH levels, and also perhaps for the field experiments in forest ponds by THOMPSON et al. (2004) and WOJTASZEK et al. (2004).

GBH can inhibit enzymes taking part in metabolism and detoxification at environmental relevant concentrations. Depletion of some of them leads to *oxidative stress* that could be related to the observed toxicity. Inhibition of enzymes takes part at sublethal concentrations of GBH. Other chronic and delayed effects are reported too. GBH are suggested to have *endocrine effects*, i.e. an impact on the sex hormone balance and disruption of the amphibian thyroid axis that can lead to precipitated or delayed time to metamorphosis. However, these can only be postulated, as the obtained results were non-significant. In addition, *immunosuppressive effects* of GBH are suggested due to elevated trematode infections in exposed tadpoles (see chapter 5.6.1.1). *Genotoxicity* (DNA damages) in tadpoles and adults has been shown, but detailed effects on individuals, offspring and especially the population level are uncertain. More research is needed in these fields.

5.6 Interactions of glyphosate and its formulations with other stressors

Single stressors can have both positive and negative effects. For example, RELYEA (2005c, 2009) has shown that insecticides can have a direct toxic effect on some predators and cladocerans, but thereby indirect positive effects on copepods and phytoplankton. With regard to amphibian larvae, there could be indirect positive effects. For example, predatory water insects can be eliminated by insecticide concentrations that do not affect tadpoles. However, indirect negative effects are also possible. When most of the cladocerans die, phytoplankton biomass increases because the remaining copepods cannot graze the smallest algae that are exclusively consumed by cladocerans. The algal bloom reduces periphyton, the tadpoles' food, due to competition for light. Therefore, tadpoles develop to be smaller and could exhibit reduced fitness at hatching or could even die due to natural drying of ephemeral ponds.

In many cases of population declines and extinctions, amphibian health is apparently not affected by a single stressor. Several studies have highlighted the importance of complexity, i.e. mechanisms underlying amphibian decline involve interactions of biotic and abiotic components (e.g. BLAUSTEIN & KIESECKER 2002; BLAUSTEIN & JOHNSON 2003; HAYES et al. 2006; GASCON et al. 2007; STUART et al. 2008).

In the following, we summarise studies which investigated impacts of GLY and its formulations on amphibians when they interact with other stressors. Table 21 summarises the results and main conclusions of available laboratory, mesocosm and field studies.

Tab. 21: Summary of main conclusions of studies on the impacts of GLY and its formulations together with other biotic and abiotic stressors on amphibians.

Stressors – study type	Amphibian species – life-stages	Main conclusions	References
Larval stages of a trematode – laboratory	*Lithobates clamitans* – tadpoles	• GLY could elevate trematode infection in tadpoles, probably due to immunosuppressive effects.	ROHR et al. 2008a
Caged predators (a newt) – laboratory	*Lithobates sylvaticus* – tadpoles	• Additional predatory stress can make GBH more deadly.	RELYEA 2005a
Predators (fish) – field	*Hyla versicolor, H. chrysoscelis* – adults	• Treefrogs can actively avoid tanks contaminated with the tested GBH and/or predatory fish.	TAKAHASHI 2007
Predators (larval salamanders) – field	*Anaxyrus americanus, Lithobates pipiens, L. clamitans* – tadpoles	• Herbicide treatment, salamander density and especially a combination of both negatively affected the fitness of the aquatic fauna in general. • Competition and predation may mediate the indirect effects of herbicides on aquatic fauna.	BRODMAN et al. 2010
Competition – mesocosms	*Hyla versicolor, Lithobates catesbeianus, L. clamitans* – tadpoles	• Competition not only reduces amphibian growth, but may also affect tadpole behaviour,	JONES et al. 2010

		physiology or endocrine function in a way that could make tadpoles more susceptible to GBH. • Effects on the aquatic community can indirectly affect tadpoles.	
Competition – laboratory	*Lithobates blairi* – tadpoles	• Higher tadpole density can increase the toxic effect of chemicals, probably due to stress.	SMITH (2001)
Mixtures of other pesticides – mesocosms	*Anaxyrus americanus, Hyla versicolor, Lithobates catesbeianus, L. clamitans, L. pipiens, L. sylvaticus* – tadpoles	• Pesticide mixtures affect the aquatic community (e.g. insecticides on predators but also zooplankton) with indirect effects on tadpoles.	RELYEA 2004, 2009
Fertiliser (ammonium nitrate) – laboratory	*Chioglossa lusitanica* – embryos	• A 'positive' effect on total length at hatching was observed.	ORTIZ-SANTALIESTRA et al. (2011)
Different pH and food levels – laboratory	*Lithobates sylvaticus* – tadpoles	• Only a marginal but non-significant effect of low food at high concentration of Vision® could be observed. • Treatments were more toxic at higher pH levels. → The	CHEN et al. 2004

		authors related the effect to structural molecular changes on the gill membrane.	
Different pH levels – mesocosms	*Anaxyrus americanus, Lithobates pipiens*, *L. clamitans*, *Xenopus laevis* – embryos and tadpoles	• Increased malformation rates were only observed in some embryos. • Toxicity was always higher at higher pH levels. → The authors supposed that non-ionised POEA, which is the predominant form at higher pH, accumulates more rapidly on the gills.	EDGINTON et al. 2004

5.6.1 Glyphosate and biotic stressors

5.6.1.1 Emerging infectious diseases

Population declines in amphibians are partly suggested to be the result of interactions of pesticide exposure and emerging parasites or pathogens (e.g. DASZAK et al. 2003; STUART et al. 2004; POUNDS et al. 2006). However, the direction and magnitude of lethal and sublethal effects of pesticides can vary (RELYEA & HOVERMAN 2006; ROHR et al. 2006a; see study of GAHL et al. 2011). Thus, there is no reason to claim that a chemical contaminant leads to either an increase or decrease in parasite prevalence or load *per se*. For a given species, certain chemicals might increase the risk of infection whereas others might do the opposite (ROHR et al. 2008a) and predictions are difficult. Sublethal concentrations of some contaminants can suppress amphibian immune defences (cf. chapter 5.5.2).

We here discuss how pesticides, in particular GBH, can increase the susceptibility of amphibians to two serious diseases.

Amphibian chytrid fungus (Batrachochytrium dendrobatidis)

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, causes an often deadly skin disease in amphibians. The so called chytridiomycosis disrupts osmoregulation or respiration across the skin of infected amphibians, releases toxins into them and probably inhibits rehydration (CARVER et al. 2010). The origin of the pathogen is still unknown and it can be found among different ecosystems on all continents where amphibians occur (FISHER et al. 2009). Chytridiomycosis can cause dramatic mass mortalities in amphibian populations up to extinction (e.g. BOSCH et al. 2001; LIPS et al. 2006; RACHOWICZ et al. 2006; SCHLOEGEL et al. 2006). The first exhaustive assessment of the conservation status of global amphibian diversity, the 2002–2004 IUCN Global Amphibian Assessment (GAA), categorised 207 species as undergoing rapid enigmatic decline, i.e. a shift to a higher IUCN Red List category between 1980 and 2004 for unknown reason (STUART et al., 2004, 2008). It has been hypothesised that these declines are all results of the pandemic amphibian chytrid fungus (SKERRATT et al. 2007; LÖTTERS et al. 2009). An outbreak of the disease is driven by multiple stressors, such as extreme climatic events (e.g. BOSCH et al. 2007; ROHR et al. 2008b). In addition, in Germany amphibians infected by the chytrid fungus can be detected nationwide. Thereby, the infection was found in all autochthonous species (OHST et al. 2011; unpublished data of the authors), except for the Alpine salamander, *Salamandra atra* (LÖTTERS et al. 2012). However, mass mortalities have not been observed yet. It is supposed that the chytrid fungus could be a potential driver of amphibian population declines in Germany, although RÖDDER et al. (2010) forecasted that future climatic conditions for the fungus could be a change for the worse.

DAVIDSON et al. (2007) conducted a study on newly metamorphosed Foothill yellow-legged frogs (*Rana boylii*). They examined the effect of carbaryl alone (0.48 mg/L), chytrid alone

(9.4×10^6 zoospores) and interactions of both stressors on survival, growth and antimicrobial skin defences. Chytrid infection reduced growth, but the authors did not find any significant effect of chytrid, carbaryl or their interactions. However, skin peptide defences, which strongly inhibited chytrid growth in vitro, were significantly reduced after exposure to carbaryl. Therefore, DAVIDSON et al. (2007) suggested that carbaryl may inhibit this innate immune defence and could increase susceptibility to the chytrid fungus.

GAHL et al. (2011) exposed Wood frog larvae to two different strains of chytrid and a GBH and the two stressors alone. The results indicate no herbicide-induced susceptibility to the fungus. Surprisingly, the higher of two herbicide concentrations reduced mortality caused by the fungus compared to animals that were only exposed to chytrid. Hence, the used herbicide seems to affect the pathogen (at least the two tested strains) more than the tadpoles.

Trematode infections

Trematode infections are of particular concern with respect to amphibian decline, as some of them parasitise amphibians and are considered to emerge due to anthropogenic environmental change (JOHNSON & SUTHERLAND 2003; SKELLY et al. 2006). Malformations in amphibians, in particular extra limbs, can be historically and recently linked to trematode infections. Besides, there is qualitative evidence that these parasite-induced malformations have increased (JOHNSON et al. 2009). KIESECKER (2002) conducted both a field experiment (with polluted waters) and a laboratory study (with atrazine, malathion and esfenvalerate) to examine the relationship between trematode-mediated link deformities and chemical contaminants in the Wood frog. The main findings were that trematode infections explain limb deformities in this amphibian and that deformities were more common at sites with agricultural run-off. Furthermore, results from the laboratory study corroborated the association between pesticide exposure and increased infection with pesticide-mediated immunocompetency.

A study with GLY is only provided by ROHR et al. (2008a). They tested the hypothesis that four common pesticides (including GLY) have lethal and sublethal effects on cercariae (i.e. larval stages) of the trematode *Echinostoma trivolvis* and its first and second intermediate hosts, a planorbid snail (*Planorbella trivolvis*) and the Green frog. They predicted that the pesticides would (i) reduce the survival of trematodes, snails and frogs (as several authors had already postulated; LAFFERTY & KURIS 1999; MORLEY et al. 2003; ROHR et al. 2004, 2006b; STORRS & KIESECKER 2004; RELYEA 2005c); (ii) decrease cercarial infectivity (cf. REDDY et al. 2004); (iii) increase tadpole susceptibility to infections (cf. KIESECKER 2002; CHRISTIN et al. 2004; BRODKIN et al. 2007). GLY concentrations in the study by ROHR et al. (2008a) were worst-case scenarios of high concentrations but still ecologically relevant (at least for North America). For this purpose, they selected the highest EEC for GLY (3.7 mg a.e./L) suggested by GIESY et al. (2000). GLY did not significantly reduce cercarial, snail or tadpole survival. However, sublethal exposure increased frog susceptibility to infections. In general, the effect of pesticides on cercarial mortality, a density-mediated effect, was two and a half times smaller than the pesticide-induced increase in tadpole susceptibility to infections, a trait-mediated effect. ROHR et al. (2008a) suggest that GLY and other pesticide exposure should elevate trematode infections in amphibians. Immunosuppression appears to be the most likely explanation for the pesticide-induced increase in tadpole susceptibility (cf. chapter 5.5.2). However, the laboratory study of ROHR and colleagues did not address all life-stages of parasites and hosts and therefore should only be regarded as preliminary and further research is needed.

Interim conclusion

It is supposed that pesticide exposure elevates the susceptibility of amphibians to diseases, most likely due to immunosuppressive effects. Conversely, pesticides may have ‘positive’ effects when they kill the pathogen more rapidly than the considered amphibian life-stage, but more research is urgently needed.

5.6.1.2 Predatory stress

For some species, additional predatory stress enhances adverse effects of pesticides on tadpoles (e.g. the insecticide carbaryl up to 46 times more lethal: RELYEA 2003). RELYEA (2005a) tested the impact of a Roundup® formulation on Wood frog tadpoles with and without the chemical cues emitted by an amphibian predator (Eastern newt, *Notophthalmus viridescens*). Adult newts were caged in plastic cups and one caged newt was added to each compartment with tadpoles that assigned the predator treatment. For further details on this study, see chapter 5.4.1. For five of six tested anuran species, survival was only affected by the GBH, but neither by predator cues nor interactions between herbicide and predator cues. Only Wood frog survival was affected by Roundup® and Roundup®-by-predator interaction. At 1.0 mg a.i./L, survival was 65% without, but only 30% with additional predator cues. There was also a trend of lower survival with predator at 0.1 mg a.i./L, but without significance. Hence, predatory stress may make GLY formulations and other pesticides more deadly to some amphibian species.

The biological mechanisms underlying this synergy still remain unknown. However, it is not caused by caged predators changing ammonia or dissolved oxygen concentrations (see RELYEA & MILLS 2001; RELYEA 2003; RELYEA 2004).

TAKAHASHI (2007) tested the effects of predator cues and herbicide presence in potential breeding ponds on the oviposition site selection of treefrogs of the *Hyla versicolor* species complex (including *H. versicolor* and *H. chrysoscelis*). Four blocks were established in an area where both species are common. Each block consisted of a central wood stand and four surrounding plastic tanks containing well water. A distance of 3 m separated each block from the next. Four treatments were randomly assigned to the tanks: control, fish (i.e. containing predatory Bluegill sunfish, *Lepomis macrochirus*), Roundup Weed and Grasskiller® (2.4 mg a.e./L) or fish cue + Roundup Weed and Grasskiller®. The tested concentration was high, about three times higher than the

$LC50_{216\text{-}h}$ (RELYEA 2005a) and about three times higher than the concentration that caused 82% mortality of metamorphic juveniles (RELYEA 2005b). Plastic tanks were initially filled with the treatments in the night when treefrogs started calling intensely. In the next morning, all pools were checked for egg masses. One block was excluded from analysis because it did not receive any eggs. There were significant effects of predatory cues, pesticide and interaction. Control tanks received nearly all treefrog eggs and the fish tanks only a few, whereas the Roundup® and the Roundup® + predatory cues tanks did not receive any eggs. Hence, TAKAHASHI (2007) concluded that treefrogs can actively avoid tanks contaminated with the tested GBH and/or predatory fishes. That appears logical from the ecological viewpoint: females prevent lethal exposures to their offspring due to breeding site selection. Larval amphibians can assess chemical cues of predatory fish and show avoidance behaviours (PETRANKA et al. 1987; KATS et al. 1988), whereas the inference that adults can also assess predatory fish cues is still unsupported. There is only indirect evidence that adults use predatory fish cues for oviposition selection (HOPEY & PETRANKA 1994). The findings of TAKAHASHI (2007) are interesting, but they suffer from a small sample size.

BRODMAN et al. (2010) conducted a field experiment on the impact of predatory stress and Accord® in 30 constructed ponds over two years. Eighty-five Northern leopard frog, 51 Green frog and 11 American toad tadpoles were placed in each pond with varying density of the predators, i.e. larval Tiger salamanders (15, 30 or 47). The authors used a completely random design. Herbicide treatment was a 5% Accord® mixture with 3% Cide-Kick II®, i.e. NPE surfactant, applied directly to aquatic weeds. Initial herbicide concentration in the ponds was about 2.0 mg a.e. and about 1.2 mg surfactant each per L. Herbicide treatment, salamander density and especially their interaction negatively affected the fitness of the anuran larvae, the present aquatic invertebrate community, but also of the salamander larvae themselves (increased mortality, reduced growth and development). BRODMAN et al. (2010) concluded that competition and predation may mediate indirect effects of the herbicide on the aquatic fauna.

Interim conclusion

Additional predatory stress enhanced adverse effects of GBH in some cases, i.e. probably in a species and formulation-specific manner. Some species are apparently able to perceive predators and, consequently, avoid these water bodies. Furthermore, it is supposed that some species can also perceive water contamination and that interactions between contamination and predatory cues exist.

5.6.1.3 Competition

Competition, especially for reduced per-capita food, is another biotic stressor that can interact with pesticides (e.g. BOONE & SEMLITSCH 2002; ROHR & CRUMRINE 2005; RELYEA & DIECKS 2008). In a community experiment by RELYEA (2009), Leopard frog (*Lithobates pipiens*) tadpoles died due to the direct toxicity of some pesticide treatments, whereas the remaining Gray treefrog (*Hyla versicolor*) tadpoles experienced greater growth due to decreasing competition with Leopard frogs on food resources (cf. chapter 5.6.2.1).

JONES et al. (2011) examined how competition affects the toxicity of Roundup Original Max® on three anuran species using outdoor mesocosms. Tanks were filled with equal aliquots of pond water containing zooplankton, phytoplankton and periphyton and supplemented with a fixed number of tadpoles (20) of American bullfrog and three different tadpole numbers (20, 40 or 60) of Green frog and Gray treefrog. This resulted in tanks with three different densities of 60, 100 or 140 tadpoles which were treated with either of four different GLY concentrations (0, 1, 2 or 3 mg a.e./L) in duplicates. Tadpole survival and biomass increase were measured throughout the experiment. The authors found that increased tadpole density caused declines in tadpole growth, but also made the herbicide significantly more lethal to one species: Whereas the LC50 values were similar across all densities for Gray treefrogs (1.7–2.3 mg a.e./L) and Green frogs (2.2–2.6 mg a.e./L), the LC50 values for American bullfrogs were 2.1 to 2.2 mg a.e./L at low and medium densities, but declined to 1.6 mg a.e./L at high densities. The large decrease in amphibian survival with increased herbicide concentration was associated with increases in periphyton abundance. Furthermore, the authors found evidence that temperature stratification leads to herbicide stratification in the water column, confirming the results of a previous study and raising important questions about exposure risk in natural systems. In conclusion, increased competition not only reduces tadpole growth, but can also make Roundup Original Max® more toxic. The herbicide became more lethal to bullfrogs at higher tadpole densities. For all species, growth declined with increasing density, probably because of less per-capita food. Given that increased density caused reduced growth but no reduction of periphyton, the data suggest that all densities of tadpoles were able to consume periphyton to a similar level. When the herbicide did cause high mortality, periphyton biomass increased suggesting a release from tadpole grazing pressure. Hence, competition not only reduces amphibian growth, but may also affect tadpole behaviour, physiology or endocrine function in ways that could make tadpoles more susceptible to Roundup Original Max®. Species-specific traits may make some species more sensitive

SMITH (2001) observed that older *Lithobates blairi* tadpoles were much more sensitive than younger ones. Regarding his study design, he supposed that this could be related to higher stress due to the higher relatively density in containers with older, larger tadpoles. That means interspecific stress could make GLY based herbicides more toxic.

CHEN et al. (2004) found a marginally non-significant effect of low food supply causing Vision® (1.5 mg a.e./L) to become more lethal (for details on the study see chapter 5.6.2.1).

To the best of our knowledge, studies on the impact on different densities of tadpoles are missing.

Fig. 15 compares the densities (tadpoles/L) and the main conclusions (effect/no effect on amphibians) of the acute toxicity studies under laboratory conditions where the relevant data were available. Although tadpole density was lowest in the study by DINEHART et al. (2010), density was very high in the study by BERNAL et al. (2009), but both studies concluded that formulations may not affect amphibians. Hence, density seems to play only a minor role in the acute toxicity experiments.

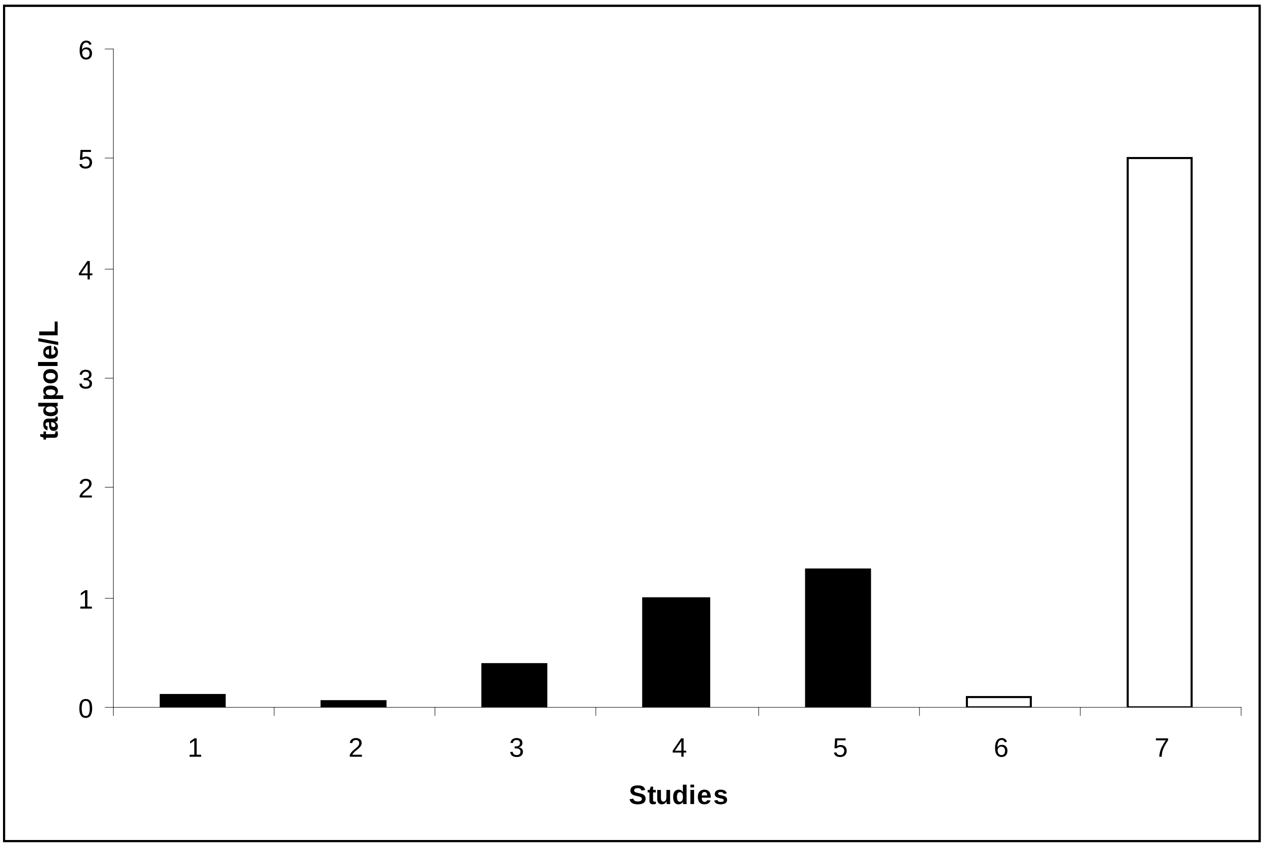

Fig. 15: Comparison of tadpole density per litre and main conclusions of selected acute toxicity studies under laboratory conditions.
Filled bars = authors suppose adverse effects on amphibians; empty bars = authors conclude no adverse effects on amphibians. Studies: (1) MANN & BIDWELL 1999; (2) SMITH 2001; (3) LAJMANOVICH et al. 2003; (4) RELYEA 2005a; (5) RLEYEA & JONES 2009; (6) BERNAL et al. 2009; (7) DINEHART et al. 2010

Interim conclusion

Especially mesocosm studies show that interactions of competition and herbicide exposure are very complex. In acute toxicity testing, density played a minor role in some experiments. However, standardised densities should be used in further experiments (e.g. one tadpole/L).

5.6.2 Glyphosate and abiotic stressors

5.6.2.1 Further environmental pollution

Pesticide mixtures

There are countless pesticides in use worldwide and numerous experiments on the impact of some of them on amphibians. Some studies have examined how mixtures of pesticides affect amphibian larvae. For example, HAYES et al. (2006) have shown that pesticide mixtures can have much greater effects on the larval growth and development of *Lithobates pipiens* than pesticides alone and negated or reversed the typically positive correlation between time to metamorphosis and size at metamorphosis. A detailed perspective on all studies using pesticide mixtures would go beyond the scope of this expert opinion. Hence, we focused on those including a GLY-based herbicide.

RELYEA (2004) examined how four commercial formulations of pesticides (the three insecticides diazinon, carbaryl, malathion and GLY) affected survival and growth of five amphibian species (cf. chapter 5.4.1). Mixtures of two pesticides occasionally caused lower survival and growth than either pesticide alone, but the effects were never larger than the more deadly of the two pesticides alone at 2 mg/L. Hence, the impact of combining these four pesticides was similar to that predicted by the total concentration of pesticides in the system.

More recently, RELYEA (2009) applied a completely randomised design consisting of 15 treatments that were replicated four times (three times for a vehicle control, i.e. ethanol). Treatments were composed of negative control (water), vehicle control (ethanol), one of five insecticides separately (carbaryl, malathion, chlorpyrifos, diazinon, endosulfan), one of five herbicides separately (GLY, acetochlor, metolachlor, 2,4-D, atrazine), a mix of the five insecticides, a mix of the five herbicides and a mix of all pesticides. Treatments were applied to mesocosms containing zooplankton, phytoplankton, periphyton and leaf litter. Each mesocosm harboured twenty tadpoles of Leopard frog and Gray treefrog as well as Wood frog tadpoles, which were about one and a half months older than the former, thereby reflecting a real-world scenario with species ovipositing at different times. Two days after the introduction of the tadpoles, pesticides were applied at nominal concentrations of 10 ppb. For most of the pesticides, this is a concentration far below maximum concentrations measured in natural water bodies. All mixture treatments were additive mixtures of pesticide such that the nominal concentration of pesticide in a mixture treatment was five or ten times higher than in the single pesticide treatments. Because Gray treefrogs take a shorter time to metamorphose, they were the first to emerge from the experiment. From day 50 up to and including day 57, each day 120 L of water was removed to simulate natural pond drying. Day 57 was defined as ‘dry pond’. All Leopard frog larvae remaining in the tank had simply not emerged due to slow growth and development. All metamorphs sampled at Gosner stage 42 were held under laboratory conditions until metamorphosis was completed (Gosner stage 46; cf. GOSNER 1960). The number of days that had passed from the start of the experiment to the completion of metamorphosis was recorded for all individuals. There was a multivariate significant effect of the treatments on all response variables (i.e. abiotic variables, abundance of zooplankton, phytoplankton and periphyton, survival, mass at metamorphosis and time to metamorphosis of the amphibians) excluding the two life-history traits of Leopard frogs. The survival of Leopard frogs was significantly reduced with diazinon, endosulfan, the mix of the five insecticides and the mix of all pesticides: 76% survived the treatment with diazinon, 16% with endosulfan and only 1% the two pesticide mixture treatments. The mix of insecticides and the mix of all pesticides significantly affected the mass at metamorphosis of Leopard frogs. Compared to the control, metamorphs were significantly smaller with diazinon and larger with endosulfan. Time to metamorphosis of Leopard frogs was only marginally affected. For Gray treefrogs, there was no effect of treatments on time to metamorphosis and survival, but there was on mass at metamorphosis. Treefrogs were larger with atrazine, the mix of insecticides and the mix of all ten pesticides. The zooplankton was also species-specific affected by the treatments. From the cladocerans, *Ceriodaphnia* sp. was sensitive to all insecticides and *Daphnia pulex* was sensitive to chlorpyrifos and diazinon. The two tested copepod species were both sensitive to endosulfan. In all cases, the mix of insecticides and the mix of all pesticides affected zooplankton. Phytoplankton increased with endosulfan and decreased with acetochlor. Periphyton was often less abundant with chlorpyrifos, diazinon and endosulfan. Furthermore, diazinon and endosulfan caused higher pH and oxygen concentrations while acetochlor caused lower pH and oxygen concentrations. All pesticides with significant effects generally had an effect both alone and in mixtures. In this experiment, GLY alone did not affect tadpole survival, mass at metamorphosis or time to metamorphosis at the applied concentration of 10 ppb (cf. chapter 5.4.2).

Interim conclusion

Mixtures of pesticides can have direct and indirect effects on aquatic communities. The extent of indirect effects depends on the applied pesticides. For example, in the mesocosm experiment of RELYEA (2009), diazinon applied alone and in mixtures caused a chain of events and resulted in 20% of non-metamorphosed Leopard frogs until ponds were dried out due to reduced periphyton mass and thus food shortage for tadpoles. Leopard frog tadpoles died due to direct toxicity of endosulfan and the remaining Gray treefrog tadpoles experienced greater growth due to reduced competition for food (cf. chapter 5.6.1.3). Other studies on the impacts of pesticides on amphibians without GLY testing also indicated indirect impacts of non-lethal pesticide concentrations on tadpoles (e.g. RELEYA & DIECKS 2008).

In summary, understanding the effects of pesticide mixtures, especially when amphibians are embedded in an aquatic community, is in its early stage and further research is urgently needed.

Fertiliser

The impact of fertilisers could play an important role in local amphibian decline and extinction. Due to their small size, most small ponds embedded in agricultural areas have no sufficient buffer capacity, so excessive application of fertilisers leads to eutrophication (SOWIG 2007). Interactions between pesticide application (including GLY usage) and fertiliser usage were recently reviewed by MANN et al. (2009). Some fertiliser can be directly toxic to amphibians. Adults and juveniles can die if fertilisers are directly applied to their sensitive and permeable amphibian skin (e.g. OLDHAM et al. 1997; HATCH et al. 2001; MARCO et al. 2001).

Furthermore, the contamination of breeding ponds is an important factor. It could be that some adult amphibians could detect and avoid high concentrations of ammonium- or urea-based fertilisers, but HATCH et al. (2001) could only observe this ability in a behavioural study when paper was impregnated with fertilisers and not evident when performed on natural soils. Amphibian embryos and larvae can be negatively affected by fertilisers (e.g. DIAMOND et al. 1993; JOFRE & KARASOV 1996; MARCO & BLAUSTEIN 1999; BOONE et al. 2005). For example, methemoglobinemia – the presence of a higher than normal level of methemoglobin in the blood – is likely to cause mortality in amphibians after nitrite exposure (HUEY & BEITINGER 1980; PUNZO & LAW 2006) while the ammonium ion in ammonium nitrate seems to have pronounced toxic effects on amphibians (e.g. JOHANSSON et al. 2001; BOONE et al. 2005). PELTZER et al. (2008) supposed that interactions between the eutrophication of breeding ponds and pesticide treatments from agriculture (and environmental factors) account for deleterious effects on survival, growth and development rate of the Neotropical treefrog *Scinax nasicus*. These effects can lead to an increase in tadpole vulnerability to parasites, erythrocytes nuclei aberrations or haemolysis. For example, ROHR et al. (2008c) suggested that environmentally relevant concentrations of atrazine in combination with phosphate-induced eutrophication can be a primary driver of larval trematode infections. However, agricultural aquatic eutrophication could also be an indirect driver in elevated parasite infections as a single stressor, mainly due to impacts on the aquatic community structure, i.e. proliferation of periphyton and the intermediate trematode snail host (e.g. JOHNSON & CHASE 2004; JOHNSON et al. 2007). Additionally, fertiliser concentrations could cause higher mortality in amphibian larvae already infected by the trematode parasite (BELDEN 2006). In particular, phosphate appears to increase exposure and susceptibility to larval trematodes by augmenting snail intermediate hosts (an indirect effect) and by suppressing amphibian immune responses (a direct effect), but mainly through complementary processes with pesticide presence (see above; ROHR et al. 2008c). In the absence of parasites or relevant pesticide concentrations, moderately elevated levels of nitrate in breeding ponds cause the proliferation of algae and macrophytes, which could be a benefit to (by providing a source of food or habitat) or have no effect on amphibian larvae (see MANN et al. 2009). However, in extreme cases eutrophication can lead to anoxic conditions in water bodies (MANN et al. 2009), proving deadly for amphibian larvae that breath using gills. Hence, the kind of fertiliser is important, but also the eutrophication of breeding ponds, regardless of fertiliser type.

We are aware of only one study on interactions between fertilisers and GBH on amphibians. ORTIZ-SANTALIESTRA et al. (2011) investigated the impact of a combination of a nitrogenous fertiliser (ammonium nitrate) and Roundup Plus® on the Gold-striped salamander (*Chioglossa lusitanica*). One hundred eighty field-collected eggs were added in 36 containers (5 eggs/container) with 50 ml water. Each container was randomly assigned ammonium nitrate[81] and Roundup Plus®[82]. Each combination of treatments was replicated four times. Solutions were renewed weekly. The experiment was terminated after 15 weeks, when all animals had either hatched or died. Mortality rate in the control was 11.3%, whereas 62.5% of embryos exposed to the highest concentration of nitrate and 31.3% exposed to the highest concentration of Roundup Plus® died. The embryonic development rate was similar across all treatments. Ammonium nitrate did not affect hatching time, length or stage. However, in another study on impacts of ammonium nitrate on urodele embryos, ORTIZ-SANTALIESTRA et al. (2007) found lower length and developmental stage at hatching. The main finding of ORTIZ-SANTALIESTRA et al. (2011) was that Roundup Plus® increased total length at hatching that was also influenced by interaction of herbicide and fertiliser. However, the authors could neither explain the biological mechanisms nor the ecological consequences of this effect. One could postulate that the observed increase in body size is related to an impact of the GBH on the thyroid hormonal balance as observed for other substances (GUTLEB et al. 2000).

Interim conclusion

Intensive fertiliser usage can bring amphibians to avoid small ponds embedded in agricultural land or reproduction to fail. In Germany, a degradation of amphibian breeding habitats, regardless of eutrophication, pesticide application or a combination of both stressors is, strictly speaking, a violation of § 44 subpara 1 point 3 BNatSchG (federal law on nature protection). Further studies on the interactions between fertilisers and GBH on different amphibian life-stages are necessary. The biological mechanisms and ecological consequences of the effect of embryonic exposure to Roundup Plus® on length at hatching have to be investigated.

Acidification of breeding sites

The laboratory, mesocosm and field studies we have cited so far all used water for controls and chemical solutions with a (starting) pH level about 7-8. However, many water bodies have considerably lower pH levels due to natural conditions or anthropogenic acidification as a result of extensive emission of acid-forming air pollutants, which mainly arise from industries, traffic or agriculture. In Germany, the problem of anthropogenic acidification of ground and surface waters was identified in the early 1980s (GEBHARDT 2007). In an acid milieu, the fertilisation of

[81] (0, 22.6 or 90.3 mg/L).
[82] (0, 2.8 or 5.6 mg/L; corresponding to 0, 1 or 2 mg a.i./L and 0, 0.24 or 0.47 mg POEA/L).

amphibian eggs can be affected, e.g. when the pH drops to 6.5 in *Lithobates pipiens* (PIERCE 1985). Furthermore, different processes in the jelly cover of amphibian eggs, hatching rates and even adults can be affected (cf. GEBHARDT 2007). In regions with natural alkaline-poor soils, a selection towards more resistant species or subpopulations could be observed (WEIßMAIR 1997). This species shift can also be postulated for anthropogenically acidified ponds. Most amphibian species completely disappear from water bodies with pH levels < 5, and more resistant species like the Common frog at pH < 4 (GEBHARDT 2007). Hence, one could conclude that sinking pH levels have *a priori* negative effects on amphibians and community composition. Two studies on interactions between GBH and pH levels are available.

CHEN et al. (2004), the first study, investigated interactions of the herbicide Vision® with two further stressors – pH and food level – on larval Wood frogs and zooplankton. All individuals were field-collected in forest ponds. Tadpoles hatched under laboratory conditions in well water (pH 6.8) and were added to the experiment at Gosner stage 25 (cf. GOSNER 1960). Zooplankton and tadpoles were studied in separate experiments. Each taxon was exposed either individual or multiple stressors. Wood frog experiments included two pH level (pH 5.5 and 7.5), two food levels (300 and 800 µg C/L) and two concentrations of Vision® (0.75 and 1.5 mg a.e./L). All treatments were replicated four times with eight tadpoles per treatment. The experiment was terminated after ten days. The survival of tadpoles was significantly reduced under both herbicide concentrations, and also below the calculated worst-case value for environmental concentrations in Canada (EEC = 1.4 mg a.e./L). The toxicity of the herbicide was significantly greater in pH 7.5. The low herbicide treatments caused complete mortality in pH 7.5, but minimal mortality in pH 5.5. In high herbicide treatments, there was no survival at any pH value, but the individuals in pH 7.5 died earlier. There were no significant effects of pH alone, and a marginally but non-significant effect of low food at high concentration of Vision®. The results of the zooplankton experiment were similar. Hence, a higher pH level increased the toxicity of the GBH in this study. CHEN et al. (2004) supposed that this increase may be caused by “the ionization state of the GLY or a threshold concentration of the herbicide that makes the pH interaction more lethal. The enhanced toxicity of the interaction is likely due to a molecular structure change on the gill membrane enhancing uptake of the toxicant”.

EDGINTON et al. (2004), the other study, examined the interactive effects of Vision® and pH on three native Canadian anurans, *Anaxyrus americanus, Lithobates pipiens* and *L. clamitans*, and the exotic *Xenopus laevis*. Like PERKINS et al. (2000), they used the standard method ‘Frog Embryo Teratogenesis Assay – *Xenopus*’, a whole-embryo test including life-stages from Gosner 8 (to 10) to 25 (cf. GOSNER 1960), but here with embryos of all four species. Furthermore, larval tests with tadpoles at Gosner stage 25 were conducted. All tests employed a rotatable design with the variables pH and Vision® concentration. The pH levels used were 4.5, 5.0, 6.5, 8.0 and 8.5. Vision® concentrations ranged from 0.1 to 20 mg a.e./L. Beside five controls with no herbicide treatment and pH 8, all other design points were replicated once. Exposure duration was 96 hours and treatment solutions were renewed every 24 hours. Due to different development rates, the embryonic tests were continued for 5 and 7 days for *L. pipiens* and *L. clamitans* respectively. In the embryo tests, *X. laevis* and *A. americanus* did not show a significant prevalence of malformations in any treatment combination. However, malformations occurred for both *L. pipiens* (mainly bent lateral tails) and *L. clamitans* (abnormal face, eye and gut development) embryos. For *L. pipiens,* an effective concentration required to cause malformations in 50% of the test organisms (EC50) could not be calculated because total mortality occurred before a 50% malformation rate could be achieved. The model for *L. clamitans* embryos produced EC50 estimates that were not different from the estimated LC50 values. According to the teratogenic index (AMERICAN SOCIETY FOR TESTING AND MATERIALS 1992) that measured the teratogenicity potential of the pH/Vision® combinations, treatments were not teratogenic to *L. clamitans* embryos at both pH levels. No malformations were observed in the larval tests. There was no significant effect on hatching time and success. Table 22 shows the $LC50_{96\text{-h}}$ values for the three species at different pH levels. The embryos of all species were more resistant than the tadpoles. An increase in toxicity with increased pH level was demonstrated for both life-stages. EDGINTON et al. (2004) explained this increasing toxicity with the toxic surfactant POEA. Because POEA is a tertiary amine blend with one fatty alkyl group and two polyoxyethylene groups attached to a nitrogen atom, gill uptake of this surfactant may occur readily and gill accumulation may be accelerated due to high proportions of the non-ionised form of POEA compared to the ionised form. The ionised form is predominant at pH 6.0 and the non-ionised form at pH 7.5. Embryos could be more resistant due to the lack or insensitivity of target organs, such as functional gills. However, EDGINTON et al. (2004) concluded that there is minimal risk of deleterious effects under real-world scenarios because the estimated LC50 values, even at pH 7.5, were below the Canadian EEC (1.4 mg a.e./L).

WOJTASZEK et al. (2004) conducted an experiment with caged tadpoles at Gosner stage 25 using *Lithobates clamitans* and *L. pipiens* in two Canadian forest wetland sites, one with a low (6.4) and one with a higher pH (7.0). Tadpoles were housed in enclosed volumes of water from the rest of the wetland. Vision® was applied twice, on 18 and 20 July of the same year, in different amounts[83]. Nearly all tadpoles died at concentrations exceeding the EEC of 1.43 mg a.e./L within one week, mainly in the first 96 hours. Tadpole mortality was greater at higher pH, comparable to the results of EDGINTON et al. (2004). The $LC50_{96\text{-h}}$ value for *L. clamitans* was 4.3 mg a.e./L at pH 6.4 and 2.7 mg a.e./L at pH 7.0. Similarly, the $LC50_{96\text{-h}}$ value for *L. pipiens* was 11.5 mg a.e./L at pH 6.4 and 4.3 mg a.e./L at pH 7.0. With the exception of *L. clamitans* tadpoles at one site, herbicide treatments did not significantly effect survival at the EEC and lower concentrations. Avoidance response was assessed within hours of herbicide treatment by gently prodding individual larvae and gauging their response as normal (larva swims away immediately) or abnormal (delayed or no response, impaired swimming ability). Herbicide concentration affected larval avoidance response in a dose-dependent manner at one wetland site. Larval growth did not

[83] Herbicide concentrations three hours after individual over-spraying of the enclosures were as follows: 0.29 mg a.e./L (2 replicates), 0.72 (2), 1.43 (3), 7.15 (2) and 14.3 (1).

significantly differ between the EEC, lower concentrations and the controls. WOJTASZEK et al. (2004) concluded that overwater uses of Vision® at concentrations that do not exceed the EEC would pose only minimal risks to amphibians. RELYEA (2011) criticised this view, as at the EEC, 50% of the tadpoles would not die, but 10-20% (compare chapter 8).

Interim conclusion

As a main conclusion, amphibian larvae in neutral and alkaline wetlands could be more affected because the acute toxicity of GBH increases with the pH level.

Tab. 22: Comparative sensitivity of embryos and larvae at different pH levels
(modified after EDGINTON et al. 2004).

Species	Life-stage	pH	$LC50_{96\text{-}h}$ [mg a.e./L]
Xenopus laevis	Embryos	6.0	**15.6**
	Embryos	7.5	**7.9**
	Larvae	6.0	**2.1**
	Larvae	7.5	**0.88**
Anaxyrus americanus	Embryos	6.0	**4.8**
	Embryos	7.5	**6.4**
	Larvae	6.0	**2.9**
	Larvae	7.5	**1.7**
Lithobates clamitans	Embryos	6.0	**5.3**
	Embryos	7.5	**4.1**
	Larvae	6.0	**3.5**
	Larvae	7.5	**1.4**
L. pipiens	Embryos	6.0	**15.1**
	Embryos	7.5	**7.5**
	Larvae	6.0	**1.8**
	Larvae	7.5	**1.1**

5.6.2.2 Habitat destruction

Global habitat change and loss associated with the expansion of industrialised agriculture is likely the single most important human activity affecting amphibian populations today (GALLANT et al. 2007). As a consequence, many amphibian populations occurring in agrarian landscapes already show negative trends even if pesticide and, in particular, GLY applications remain out of consideration. For example, in Germany the agricultural landscape was characterised by an enormous diversity of landscape components and habitats before the industrialisation of agriculture occurred after World War II. In the past, extensively used areas and a multitude of small ponds harboured large amphibian populations. Hence, agrarian industrialisation has led to massive setbacks to amphibian populations (GÜNTHER 1996). This is also true for other industrial or 'developed' countries like the USA.

In South America, a significant agricultural transformation has taken place more recently, during the 1990s. Taking Argentina as an example, agriculture could spread not only in the Pampas region, but also over additional areas with high biodiversity, mainly driven by the adoption of GM crops and the no-tillage system. Important ecoregions like the Great Chaco and the Mesopotamian Forest have been made accessible. In the last ten years or so, especially intensive soybean production has displaced more than 4,500,000 ha of land previously dedicated to other land use. Furthermore, an intensive usage of fertilisers is required in most of these areas

(PENGUE 2004).

Interim conclusion

Recently, agriculture spread in less profitable areas. This is caused, for instance, by the increasing cultivation of energy crops. Furthermore, some GM crops enable farmers to use less profitable areas, but only with intensive use of fertilisers and pesticides. This trend represents a new threat for amphibian populations but also other organisms and could be compared with the intensification of agriculture after World War II in Europe.

5.6.2.3 Global change

Anthropogenic climate change

Several impacts of potential anthropogenic climate change on amphibians are being discussed today. For example, an increase in infections with the amphibian chytrid fungus could be postulated. Different authors stated strong correlations between outbreaks of the disease chytridiomycosis and extreme weather events (e.g. BOSCH et al. 2007; ROHR et al. 2008b). CHEN & McCARL (2001) forecasted that climate change could increase pesticide applications in some cultivations and, therefore, could increase adverse effects on amphibians. Elevated temperatures could lead to earlier metamorphosis and decreased size and fitness at metamorphosis (JAKOB et al. 2002). However, this observation may depend on the species and the region in which the populations occur (BEEBEE 1995). Furthermore, amphibian reproduction could take place earlier in the year. For example, in some German regions amphibian species are already reproducing on average three weeks earlier in the year than in previous decades, even in agrarian regions (MÜNCH 1999). In the future, different species and/or life-stages could be directly exposed to agrochemicals than are exposed today (assuming that agrarian practices will not change in the same rate; cf. chapter 6). Particular studies on interactions between climate change and GBH are lacking.

Solar radiation

Solar radiation can alter the hatching success of some but not all species studied, e.g. in the Nearctic Cascade frog (*Rana cascadae*) or the Common toad, *Bufo bufo* (LIZANA & PEDRAZA 1998). However, even if UV-B radiation does not reveal significantly negative effects in the hatching success (e.g. as in the Nearctic Pacific chorus frog, *Hyla regilla,* or the European Natterjack toad, *Epidalea calamita*), increased UV-B radiation can still lead to sublethal effects in species (BLAUSTEIN et al. 1994b; LIZANA & PEDRAZA 1998). Several laboratory studies have illustrated that solar radiation has an effect on the growth and development of tadpoles rather than hatching success, as demonstrated in the Nearctic Plains leopard frog (*Lithobates blairi*) or the

Common frog (*Rana temporaria*) (SMITH et al. 2000; PAHKALA et al. 2001). Differences of within-species tolerance were also observed. For example, larval stages of the Long-toed salamander, *Ambystoma macrodactylum* from North America were more sensitive to the effects of solar radiation at lower elevations above sea level than those from higher elevations (BELDEN & BLAUSTEIN 2002). In addition, it has been suggested that differences among amphibian species may be related to species-specific adaptations. In field experiments, fertile eggs of the most UV-resistant amphibians showed higher activity of the enzyme CPD-photolyase than those of more susceptible species (BLAUSTEIN et al. 2003). This is related to the fact that the major process by which amphibians remove or repair UV-damaged DNA is through enzymatic photoreactivation, exploiting this enzyme (FRIEDEBERG et al. 1995). BLAUSTEIN et al. (2001) pointed out that embryonic amphibians of declining species possess less photolyase activity than those of apparently intact species. BLAUSTEIN et al. (2003) reviewed studies on the effects of elevated UV-B radiation alone and in combination with pesticide exposure and summarised that synergistic interactions of UV radiation with contaminants can enhance the detrimental effects of the contaminant and UV radiation.

PUGLIS & BOONE (2011) tested the effect of additional UV radiation on Green frog tadpoles, which were exposed to GLY isopropylamine salt or Roundup Original® (and six other a.i. and formulations). The authors found that either the presence or the absence of UV radiation affected the survival of tadpoles in five of seven pesticides tested because UV radiation increases the rate of breakdown into by-products that are more toxic or less toxic than the original chemical. Although the effects of the tested GBH, concentration, the interaction of the GBH x concentration, and the interaction of the GBH x concentration x UV on tadpole mortality were significant over time, mortality was low in all treatments except for high concentrations[84]. The authors did not observe large differences in mortality between UV treatments, and at the EEC of 1.43 mg a.e./L, no mortality has been observed at all in this study.

Interim conclusion

Particular studies on interactions of global change stressors – especially climate change – with GBH are widely lacking. Hence, further research is urgently needed.

[84] Tested concentrations were 0, 0.625, 1.25, 2.5, and 5 mg a.e./L.

5.6.3 Interim conclusion and discussion for chapter 5.6

Laboratory studies usually use single stressors in standardized designs (e.g. standardized exposure of animals to one herbicide) to obtain causative results. In the real-world, amphibians (and other organisms) are faced with multiple stressors. With regard to the results of the so far conducted studies with tadpoles, the presence of multiple stressors apparently can have positive effects on tadpoles in a minority of cases (e.g. directly via the reduction of chytrid zoospores rather than toxic effects on tadpoles by a formulation or indirectly by the elimination of predators due to insecticides), but negative effects prevail and in most cases, adverse effects are enhanced by the presence of multiple stressors. However, this is logical and also true for most other contaminants and not only for GLY and its formulations. In a general view, it has to be pointed out again (cf. chapter 2) that most likely habitat destruction and other threats such as emerging infectious diseases have greater impacts on amphibian populations as environmental contaminants, but this does not necessarily apply to amphibians in intensively used agricultural areas. Furthermore, a recent spread of agriculture can be observed, for instance due to the increasing cultivation of energy crops, which leads in many cases to ploughing up off grassland.

6. Pathways of exposure to different amphibian life-stages

Intensively used agricultural areas are predominantly not suited to be amphibian habitat because they are often too dry and hot (especially for juveniles), sufficient food is absent for most species (mainly due to the use of insecticides), agricultural work processes are destructive and pesticides and fertilisers are being applied in greater varieties (SOWIG 2007; MANN et al. 2009). Amphibians are rarely detected directly in fields. Notwithstanding, even today agricultural landscapes provide a habitat for many amphibian species (MANN et al. 2009). Remaining major terrestrial habitats of amphibians in agricultural areas are in extensively used structures (such as bosks or fallows) between the intensively used fields (SOWIG 2007; BERGER et al. 2011). Furthermore, water-logged areas in fields can give valuable amphibian habitat in summer due to large food supply and a favourable microclimate (BERGER et al. 1999, 2011).

Breeding sites within agrarian landscapes include – apart from different kind of water bodies near fields – ephemeral water bodies such as vernal pools or puddles (that can also be found directly in fields) and especially drainage channels of acres (SOWIG 2007; BATTAGLIN et al. 2009; MANN et al. 2009). These small and ephemeral water bodies are especially exposed to agrochemicals, because they are usually not protected by pesticide label requirements for no-spray buffer zones (e.g. MANN et al. 2003; THOMPSON et al. 2004; BATTAGLIN et al. 2009). Because of their small size they will rapidly become eutrophic and have higher concentrations of pesticides compared to larger water bodies (SOWIG 2007).

Furthermore, amphibians are often forced to pass agricultural land when migrating to and from their breeding sites and from their summer habitats to their winter quarters.

We consider different potential pathways of exposure to GBH within conventional and HR crop systems (cf. Fig. 16). They differ in amount and time period of exposure:

(1) Adults and juveniles may be directly over-sprayed during migrations (high amount – short period).

This can occur before sowing in no-tillage farming, which is mostly the case for HR crops, but also for conventional crops; it can occur several times during growth of HR crops and during desiccation of several conventional crops (cf. DILL et al. 2010).

Most of the reproductive adults of an amphibian population in Germany are migrating from their winter quarters to their breeding sites and – when located in agricultural landscapes – they are occasionally forced to pass fields. In Germany, most adults of 'explosive breeders' migrate in spring within a short period of time (March-April) (see WELLS 2007). They migrate during the night and also in the daytime, when pesticides are applied. Since amphibians prefer wet weather (e.g. GÜNTHER 1996) and pesticides are usually applied when the weather is dry, the risk of direct-over-spraying should be limited. However, in areas with large fields, animals may rest on them (see below) and get over-sprayed or indirectly exposed. Also 'prolonged breeders', which occur in Germany (see WELLS 2007), migrate from their winter quarters to their breeding sites, but furthermore several times from their breeding sites to their summer habitats ('prolonged breeding time'; April-July/August: GÜNTHER 1996). This enhances their risk of getting over-sprayed directly, but again migration usually takes place when the weather is wet (e.g. when temporary pools were filled by rain water).

BERGER et al. (2011) calculated how likely it is that different amphibian species encounter herbicides on their way from the winter quarters to the breeding sites. The so called temporal coincidence of organisms and herbicides considers direct contact, when over-spraying and migrating occur at the same time, and indirect contact via the soil or plants, when amphibians migrate over a recently treated field. For the latter, Berger et al. (2011) considered specific DT50 values (i.e. the time, when the initial concentration of a substance is halved) for their calculation. Especially Common spadefoot toads (*Pelobates fuscus*) were affected in this way, because this species spends most of the year directly on fields. The temporal coincidence of Fire-bellied toads (*Bombina bombina*), Moor frogs (*Rana arvalis*) and Crested newts (*Triturus cristatus*) was up to 50% with spring crop cultivation and lower with winter crop cultivation. This is because winter crops require less herbicide applications and spring crops are treated with herbicides with a high DT50 value. In the area studied by BERGER et al. (2011), spring crops were treated with herbicides with high DT50 values (up to 315), while GBH have much lower DT50 values (e.g. 30-40 for Roundup UltraMAX®, www.roundup.de). Hence, temporal coincidence of herbicide applications and migrating amphibians in spring is given, but indirect contact (via plant material and soil) is more likely than direct over-spraying (see exposure pathways (2) and (3) below).

Terrestrial summer habitats of amphibians in agricultural landscapes – where the activity of the animals is mainly limited to feeding – are nowadays mainly situated in extensively used structures (see above). Amongst others, this is because in intensively used fields their food source is often reduced by insecticides. However, when pest insects rapidly emerge, this may attract amphibians to intensively used fields as well. In addition, water-logged areas with large food supply and favourable microclimate are often accepted. BERGER et al. (2011) especially found juveniles of up to seven species in such water-logged areas.

Due to the prolonged breeding time, not all juveniles of 'prolonged breeders' metamorphose at the same time while newly metamorphosed juveniles of 'explosive breeders' migrate in masses from their birthplace in late summer. Hence, in a worst-case scenario a substantial part of the offspring of 'explosive breeders' may be threatened by herbicide applications. In the case of GBH, especially the method of desiccation of conventional crops – which is applied in summer before harvest – seems to be a threat to migrating 'explosive breeders'. While metamorphs of both 'explosive' and 'prolonged breeders' generally wait for wet weather to leave their birth places (GÜNTHER 1996), BERGER et al. (2011) found three different behaviour patterns for juveniles, but also for adults: (i) animals passed agricultural areas quickly (within a single night), (ii) animals avoided unstructured areas and migrated "around", (iii) animals found suitable shelter in fields and rested. BERGER et al. (2011) stated highest temporal coincidence of (general) herbicide applications for winter crops and migration of Crested newts and Fire-bellied toads from their breeding sites in late summer/autumn. This is related to the lifestyle of both juveniles and adults of the two species: they spend most of the summer in water and they need a long period to leave their breeding sites. Hence and as outlined, the risk of direct over-spraying is again a function of the local conditions and the respective species.

In autumn, all amphibian species in Germany migrate from their summer habitats to their winter quarters, but – some individual variations are allowed – mainly under wet conditions. Using radiolabelling of individuals, BERGER et al. (2011) showed that Common toads passed agricultural areas with bare grounds quickly, but rested if they found plant material or rodent tunnels to hide. Hence, depending on the structure of the landscape, some individuals may be exposed to GBH applications in autumn (e.g. before winter crop cultivation).

Similar to summer quarters, also winter quarters of amphibians are mainly found in extensively used structures between the fields, e.g. rock fragment piles (BERGER et al. 2011).

In no-tillage farming, decaying plant material that is left on the fields could provide suitable shelter for amphibians and attract them in this way while at the same time put them at risk through the pre-sowing application of GBH (and other broad-spectrum herbicides). At present, this is only a hypothesis which should be tested in practice.

In conclusion, direct over-spraying of amphibians with GBH is possible in conventional and HR crop farming, but indirect contact is more likely. Because amphibians are mainly active during wet conditions, the risk of direct over-spraying is limited as herbicides are usually applied under dry conditions. In general, the risk of direct over-spraying depends on the local conditions (especially the cultivated crop and the condition of the field ground) and of the habits and lifestyles of the amphibian species.

(2) *Adults and juveniles may have contact with leaves with adhered herbicide (low amount – medium period).*

One main purpose of surfactants is to enable herbicides to adhere to the surface of leaves. As a result, some herbicide residues are present right there and on other plant parts as well. With regard to the above mentioned findings of BERGER et al. (2011), contact with contaminated plant material (and soil; see below) is more likely than direct over-spraying. However, particular studies of herbicide uptake via contaminated plant material are lacking (e.g. BRÜHL et al. 2011).

(3) *Adults and juveniles may have contact with contaminated soil (low amount – medium period).*

GLY is rapidly adsorbed onto soil (RUEPPEL 1977), which often limits the contamination risk of amphibians, but phosphate concentration is the most important factor determining the amount of GLY adsorbed due to limited number of adsorption sites and, furthermore, the soil type is important (BOTT et al. 2011; cf. chapter 3.4.1). Hence, it depends on local soil conditions how much GLY is adsorbed. Furthermore, GLY can also be remobilised by phosphate fertilisers (BOTT et al. 2011), but amphibians that are present on fields during fertilisation are likely more affected by the fertiliser than by the remobilised GLY. Conversely, remobilised GLY can be laterally transported in the soil so that GLY can also occur nearby (not only on) fields (BOTT et al. 2011). However, it is unknown whether the resulting GLY concentrations are a threat to amphibians.

(4) *Embryos, tadpoles, metamorphs and reproducing adults may be exposed to the current concentrations at breeding sites (low to high amounts – short to long period).*

Breeding sites can be contaminated due to pesticide drift, wind erosion of contaminated soil, run-off and direct over-spraying. Possible GLY concentrations in aquatic amphibian habitats are discussed in chapter 5.2. At worst-case conditions, all life-stages are affected. However, the actual concentrations of GLY and especially added substances (surfactants) in amphibian habitats in the agricultural landscape of Germany are unknown. Even the worst-case EEC is only calculated for pesticide drift during application (BVL 2010c) because contamination via wind erosion of contaminated soil or run-off is site-specific.

(5) *Adults and juveniles may be exposed in their nearby terrestrial habitats, due to pesticide drift, wind erosion of contaminated soil or contaminated run-off (low to medium amount – low to medium period).*

DAVIDSON (2004) showed that pesticides can drift – due to ‘favourable’ weather conditions – even into mountainous areas in California, kilometres away from the areas where the pesticides were applied. However, application practices in North America and Germany can remarkably differ (e.g. aerial applications). One could discuss about drift rates for pesticides which may contaminate terrestrial habitats of amphibians, but this is again a function of local conditions and no particular data are available. The same applies to wind erosion of contaminated soil and run-off contaminated with pesticides.

(6) Adults and juveniles feed on contaminated invertebrates etc., likewise larvae may feed on contaminated sediment or detritus (low to medium amount – medium period).

Currently, studies on the impact of contaminated food on amphibians are largely lacking. McCOMB et al. (2008) intraperitoneally injected Rough-skinned newts (*Taricha granulosa*), Ensatiana salamanders (*Ensatina eschlotzii*), Tailed frogs (*Ascaphus truei*), Pacific giant salamanders (*Dicamptodon* ensatus) and Western red-backed salamanders (*Plethodon vehiculum*) with technical GLY isopropylamine salt. $LD50_{96\text{-}h}$ values were always over 1,000 mg/kg. Rough-skinned newts were also gavage fed and the $LD50_{96\text{-}h}$ was four times higher than those observed by injections. Hence, McCOMB et al. (2008) concluded no risk under real-world scenarios, but they only tested GLY isopropylamine salt that is known to be less harmful than the formulations used in the real-world (e.g. MANN & BIDWELL 1999). Studies on food that is contaminated with GBH are lacking.

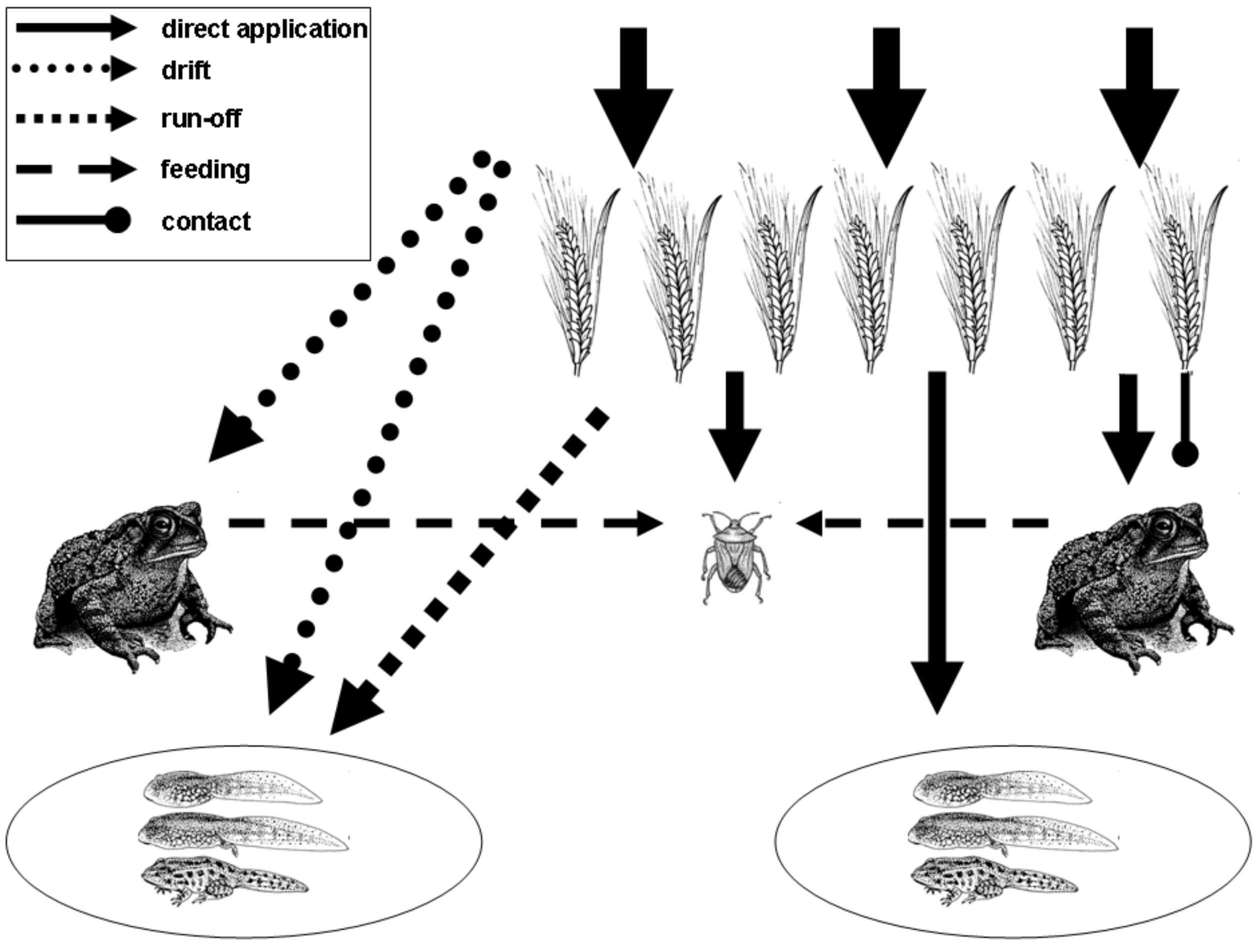

Fig. 16: Examples of potential exposure pathways of GBH to amphibians due to conventional and HR systems.
On the left are life-stages in habitats nearby agricultural land; on the right are life-stages occurring in fields or in water bodies associated with fields. (Note: The adult toad, the frog tadpoles/metamorphs but also the bug as food should only serve as an example of an "agrarian community".) Part of the figure is based on drawings from http://commons.wikimedia.org

General aspects

Amphibians can also be exposed to AMPA concentrations due to erosion of contaminated soil, run-off and degradation of GLY, but particular studies are lacking. Due to the observed long-term leaching and accumulation of AMPA (cf. chapter 5.2), this main degradation product seems to be equally important for environmental risk assessments than GLY itself.

The results of EDGINTON et al. (2004), HOWE et al. (2004) and ORTIZ-SANTALIESTRA et al. (2011) suggest that embryos are more resistant than tadpoles. Hence, exposure during the larval phase seems to be more relevant.

However, when could amphibians be exposed to GBH? Firstly, most GM crop systems use no-tillage practices before sowing (e.g. PENGUE 2004). Here, but also in conventional no-tillage farming, GBH are applied shortly after or before sowing to combat weeds without tillage. In contrast to the conventional agriculture, non-selective herbicides can be used later in cultivation in the HR system, e.g. in HR sugar beet when seedlings already have 4-6 leaves. In conventional farming, herbicides are mainly applied during the seedling stage (MÄRLÄNDER & VON

TIEDEMANN 2006). Normally, in the time period from sowing to the 4-6 leave-stage, GBH are applied a second time (GRAEF et al. 2010; SCHÜTTE & MERTENS 2010). However, GLY can further be applied during large parts of the remaining cultivation period, and the following aspects are relevant in this context: (i) Multiple applications of broad-spectrum herbicides are supposed to not significantly weaken the yield in GM crop cultivations (e.g. BEIßNER 2000; MÄRLÄNDER & VON TIEDEMANN 2006), (ii) the cost of GLY dropped dramatically after the patent of Monsanto expired in 2000 (DUKE & POWLES 2008), and (iii) resistant weeds due to a lack of crop rotations have to be combatted with selective herbicides alongside higher amounts of non-selective herbicides (DUKES & POWLES 2009). Run-off in nearby ponds then mainly occurs during the first heavy rainfalls after an application (e.g. HENKELMANN 2001).

Crop systems, seedtimes and correspondingly the amount of herbicides and time at which herbicides are applied differ. With regard to the crop system, MORTENSEN et al. (2008) named application rates up to 4.2 kg a.e./ha for no-tillage use (conventional and HR system) and up to 1.74 kg a.e./ha for in-crop use, i.e. during growth of HR crops. Also, the kind of formulation and its concentration varies according to the application date. For example, in Argentinean HR soybean crops Roundup Ultra Max® can be applied at 30 to 45 days after emergence at a concentration of 1.1 kg a.e./ha, whereas other formulations are applied before the emergence at higher concentrations (LAJMANOVICH et al. 2011). Hence, potential application periods have to be assessed specifically with respect to site, cultivation and region (see also Table 24 for potential impacts on amphibians in German conventional agriculture).

It has to be considered that outside the agrarian landscape, there are further ways how amphibians can get exposed to GBH, e.g. contaminated run-off from over-sprayed roads and railways (WOOD 2001), aerial application at forest sites (e.g. EDGINTON et al. 2004; THOMPSON et al. 2004) or private use. For example, DINEHART et al. (2009) found two GBH for private use extremely toxic for the Great Plains toad (*Anaxyrus cognatus*). While the authors concluded no risk in practice, because the two formulations are not applied in agricultural areas, where the Great Plains toad prevails, exposure can be assumed since this species also tolerates urban conditions (KRUPA 1994) where the two GBH may be used.

Interim conclusion

Amphibians can be exposed to GBH via different pathways. Whether an amphibian species is exposed at all, to what extend and by which pathway depends on the site, the cultivation system, the region and the amphibian life-stage. No general risk can be named, but compared to conventional cultivation, HR systems allow GBH (as well as other broad-spectrum herbicides) to be applied later and potentially several times. Hence HR systems have broader potential impacts of GBH on the varying amphibian species with respect to considered life-stages and the extent of exposure. However, it has to be kept in mind that in conventional agriculture, always different selective herbicides are applied before crop growth (additionally to GBH when no-tillage farming is practiced). In HR systems, selective herbicides have to be only applied if weed resistances are present in the cultivation area.

6.1 Germany, as a special case

Germany can be used as example for potential exposure to GBH within conventional agriculture, as HR crops are not approved and cultivated yet. In the last decades, no-tillage farming increased in Germany and was even funded by some German states for the purpose of soil protection (RAUBUCH & SCHIEFERSTEIN 2002). Currently, no-tillage conventional farming appears to be the main direct pathway for amphibians to get exposed to GBH. Because there is, for example, spring and winter crops, time periods for application in no-tillage farming differ with the sowing date (see Table 20). Again, specific exposure risks mainly depend on the site, i.e. the local conditions, cultivated crops and annual cycles, as well as the habitats of amphibians and the species that occur. Migrating amphibians occasionally rest during the day in hiding places in fields, e.g. under crop residues, between catch crops or weeds (see the results of BERGER et al. [2011] above). At a rough estimate, early 'explosive breeders' among the amphibians can be directly exposed to GBH during their migration to breeding sites (no-tillage before spring crop cultivation; March; Table 24). However, cultivation of winter crops is more common in Germany today than cultivation of spring crop (STATISTISCHES BUNDESAMT 2011; see Table 23). No-tillage farming of maize (seedtime and potential GLY application mid-April to beginning of May) can affect early breeders when migrating back to their summer habitats. In contrast, GBH use in no-tillage maize cultivation can have impacts on adults of later, 'prolonged breeders' when migrating to their reproduction sites (Table 24).

Metamorphs, especially of 'prolonged breeders', can directly be over-sprayed when they have to migrate across fields from their hatching sites in late summer and early autumn (no-tillage in winter crops) (Table 24). Furthermore, all life-stages of almost all species found in Germany can be affected when migrating from their summer habitats to their winter quarters. If they have to cross fields in autumn (mainly in October), they can be exposed to GBH in no-tillage winter crop cultivation (Table 24).

Breeding ponds, which are embedded in the agrarian landscape, can be contaminated via direct over-spraying, pesticide drift, wind erosion of contaminated soil and run-off and this is mainly due to herbicide use in no-tillage farming of spring crops and maize. Early amphibian life-stages, which arise from these breeding ponds are probably more affected from spring crop farming and less from winter crop farming because the applied herbicide (GLY and most likely also the added substances) with no-tillage winter crops will be degraded until spring, when the early life-stages develop, which is much different for spring crops.

At the current state, detailed information on concentrations of GLY, added substances and AMPA in small ponds within the agrarian landscape are lacking. Likewise the impact of no-tillage farming on amphibians is unknown. It can be assumed that crop residues, catch crops and weeds on no-tilled fields could especially attract amphibians and, therefore, increase their risk to come into contact with GBH. Conversely, there is some information on simultaneous ploughing and amphibian migration with a very high mortality rate (e.g. DÜRR et al. 1999). With regard to the population viability, one might argue that it does not matter if individuals are killed by chemicals in no-tillage farming or by ploughing as long as both farming methods are equally lethal.

Tab. 23: Seedtime of the five most important crops, which are cultivated on nearly 75% of all agricultural land in Germany in 2011
(STATISTISCHES BUNDESAMT 2011).

Crop	Cultivated land [in 1,000 ha]	Seedtime
Winter wheat	3,260.9 (26.8%)	End of Sep – begin of Dec
Maize	2,520.3 (21.2%)	Middle of Apr – begin of May
Winter canola	1,312.7 (11.0%)	Middle of Aug – begin of Sep
Winter barley	1,185.6 (10.0%)	Middle of Sep – middle of Oct
Winter rye	615.4 (5.2%)	Middle of Sep – middle of Oct

Legend: In no-tillage conventional farming, GBH are applied shortly before or after sowing; hence, the seedtime indicates the period in which GBH may be applied.

Tab. 24: Raw scheme on potential temporal coincidence of GBH application in no-tillage farming and annual activities of selected amphibian species in Germany
(based on data from LAUFER et al. [2007] and STATISTISCHES BUNDESAMT [2011]).

GBH application in no-tillage farming of important crops	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Winter wheat												
Maize												
Winter canola												
Winter barley												
Winter rye												
Winter grain												
Spring grain												
Amphibian activity [a] (alphabetical order)												
Bombina bombina [b]												
Bombina variegata [b]												
Bufo bufo												
Epidalea calamita [c]												
Hyla arborea												
Ichtyhosaura alpestris												
Lissotriton helveticus												
Lissotriton vulgaris												
Pelobates fuscus [d]	o	o				o	o	o	o	o	o	o
Pelophylax kl. esculentus [e]												
Pelophylax lessonae [e]												
Pelophylax ridibundus [e]												
Pseudepidalea viridis												
Rana arvalis												
Rana dalmatina												
Rana temporaria												
Triturus cristatus												

Legend:
One list indicates important conventional crops and the other list indicates amphibian species occurring in agrarian landscapes.

Period for potential GBH application in no-tillage farming

Adults are crossing fields while migrating to, reproducing in and migrating from breeding sites to their summer habitats. Eggs, embryos and larvae reside in the aquatic habitats, which can be on or nearby fields.

Metamorphs are leaving hatching sites.

Most individuals are migrating to their winter quarters.

❍ Many *Pelobates* individuals have their summer and winter habitats directly in fields.

a Only amphibian species occurring in agrarian landscapes of Germany.

b *Bombina* metamorphs generally stay at their hatching sites for some time; dispersal may occasionally occur during rainfalls; adults are semiaquatic, i.e. they stay at the breeding sites over most of the vegetation period, and return to their winter habitats in September to October.

c	*Epidalea* larvae can rapidly metamorph (at average within 1-2 months), i.e. the first metamorphs may leave the hatching sites around end of April. On the other hand, this prolonged breeder can reproduce until late in the year.
d	Some *Pelobates* larvae overwinter in permanent ponds and in some years there could be a second breeding time in August.
e	*Pelophylax* metamorphs stay at the hatching sites for some time, but adults are even more aquatic than juveniles and often overwinter in permanent ponds.

In general, it is widely unknown how many amphibian populations actually occur in agrarian areas in Germany. Official data on the distribution of common species are hardly available because there is no commitment to map them. The Habitats Directive foresees to report on the distribution of strictly protected species only, but one can assume that (especially small) populations in agrarian landscapes are often overseen. Especially, it remains widely unknown to what extent amphibians reside directly on fields (greatest exposure risk to GBH). For example in the state of Baden-Württemberg, practically no amphibians were reported directly on fields. Only 2% of all detections of the Common toad (*Bufo bufo*) and about 10% of the Natterjack toad (*Bufo [Epidalea] calamita*) were made on acres. Even detections of the Common spadefoot toad (*Pelobates fuscus*) seem to be unusual in Baden-Württemberg (SOWIG 2007). However, *Pelobates fuscus* (besides dunes and 'cultural steppe landscapes' as heathlands, sand- and clay pits or military areas) are often found in acres with sandy soil (especially asparagus, vegetable, potato or maize cultivations) where they live subterrestrially most of the year (e.g. NÖLLERT & GÜNTHER 1996). Therefore, this species seems to be the amphibian species in Germany with the highest risk of agricultural collateral damages via tillage or exposure to agrochemicals.

Conversely, water-logging directly on fields can lead to temporary pools that are sometimes used for reproduction by Common frogs (*Rana temporaria*) in spring or Natterjack toads in summer (SOWIG 2007). In the Upper Rhine plain, FLOTTMANN & LAUFER (2004) reported reproduction of Common frogs, Common spadefoot toads, Green toads (*Pseudepidalea viridis*) and European treefrogs (*Hyla arborea*) in such temporary pools. In this area, European treefrogs even prefer such water bodies.

There are also some specific and long-term studies on the population dynamics and migration behaviour of amphibians in agrarian landscapes in Germany (e.g. KNEITZ 1998; HACHTEL et al. 2006; BERGER et al. 2011). However, these valuable and extensive studies only refer to restricted areas. Conclusions for similar areas could be postulated, but a widespread nationwide survey on amphibian populations within agrarian landscapes is lacking.

Interim conclusion

In Germany, mainly winter crops and maize is cultivated today. No-tillage applications in winter crops may affect (i) freshly metamorphosed individuals (especially of 'prolonged breeders') that have to pass fields in late summer and early autumn and (ii) all amphibian life-stages that migrate from summer habitats to winter quarters. Conversely, no-tillage farming of maize may affect (i) adults of early, ‘explosive breeders’ when migrating back to their summer habitats, (ii) adults of later, ‘prolonged breeders’ when migrating to their reproduction sites and (iii) aquatic life-stages when breeding sites are contaminated. Again, wild amphibian’s risk to no-tillage conventional farming depends on local conditions, cultivated crops and occurring species.

Furthermore, desiccation of several conventional crops is practiced in late summer. This offers similar risk like no-tillage applications in winter crop cultivation.

There is practically no information on general pesticide concentrations in small water bodies within agricultural areas and very little information on amphibian populations that persist in agricultural areas in Germany.

7. The agricultural system of genetically modified crops and possible further impacts on amphibians

GBH applications and their potential drawbacks have been pointed out in chapter 5. However, there are yet more indirect impacts of the HR crop system on amphibians left to discuss.

Exclusive no-tillage farming in HR crop systems

HR crops are often cultivated without or with low tillage. As mentioned before, no-tillage farming is also practiced with conventional crops, but the number and timing of applications of total herbicides differ (see chapter 6). We are aware of the problem of ploughing, which can lead to next to 100% mortality for amphibians (DÜRR et al. 1999). The question of whether no-tillage farming is less harmful for migrating amphibians cannot be answered here, as specific studies on this issue are lacking (cf. chapters 6 and 9). It won’t matter if amphibian populations, which persist in agrarian landscapes, suffer ‘mechanical death’ (due to ploughing or other mechanical cultivation methods) or ‘chemical death’ (due to applications of GBH or other herbicides). No-tillage farming could have positive effects on amphibians because temporarily uncultivated land acts as suitable terrestrial habitat or resting place for migrating individuals (see BERGER et al. 2011). It might have negative effects as well because animals are attracted by uncultivated land and they are more likely to be harmed by total herbicides applications associated with the farming method.

Potential increase in fertiliser usage

There is another potential indirect effect of the HR crop system on amphibians to consider, namely agricultural practices which aim to combat GLY-induced underperformance of crops. GLY usage can impact the nutrient status of the soil and might impair the nutrient availability for plants, their fitness and yield. When this is counteracted by increased use of fertilisers or pesticides, it can adversely affect amphibians (see chapter 5.6.2.1) and enhance impacts. Here is a description of the involved processes in the soil: certain microbes are inhibited upon GLY application, among them beneficial ones that fix nitrogen or reduce manganese to a plant available form (→ more fertiliser) and also microbes that antagonise some plant pathogens (→ more pesticides). Other microorganisms take over and profit, such as plant pathogenic *Fusarium species* (filamentous fungi) and bacteria that oxidise manganese to a form no longer available to plants. Taken together, GLY shifts the composition of the soil microflora; it inhibits beneficial features of the soil and promotes detrimental ones. This picture about how GLY impacts the soil is based on the following articles: (i) KREMER & MEANS (2009) reviewed literature concerning GLY-induced plant pathogen activity (i.e. species of *Fusarium*) in the rhizosphere and negative impacts on soybean nodulation. They found that the pseudomonad component, which antagonises fungal pathogens, was decreased and that the proportion of Mn-oxidising bacteria was increased. When manganese is oxidised (Mn^{4+}), it is no longer available to plants. (ii) MERTENS (2011) also shed light on the interactions between GLY and soil microorganisms. Principal relevant findings were the negative effects on the nitrogen fixing bacterium *Bradyrhizobium japonicum* and pseudomonads, which reduce manganese to Mn^{2+} and make it available to plants. Furthermore, both overviews arrived at the conclusion that beneficial fungi like mycorrhiza or entomopathogenic fungi (= that kill harmful insects) could be adversely affected by GLY. (iii) More recently, ZOBIOLE et al. (2010) investigated the effect of HR soybean cultivation on the nitrogen fixing bacterium *Bradyrhizobium elkanii* in two different soils. In the greenhouse, non-HR soybean, HR soybean with a non-GLY herbicide and HR soybean with two different GLY doses were planted. The authors analysed photosynthesis and mineral nutrients in leaf tissue of all groups and found much lower chlorophyll concentrations in plants treated with GLY. Furthermore, highest nutrient levels were found in leaves of non-HR soybeans even when compared with HR soybeans not treated with GLY. Taken together, two factors influenced the nutrient efficiency of HR soybeans: (a) GLY-resistant genes and (b) the applied GLY. Insertion of the transgene alone reduced the level of both macro- and micronutrients, especially calcium, magnesium, zinc, manganese and copper. Nutrients were reduced further when GLY was applied. GLY reduced all macro- and micronutrients (except nitrogen and iron in one soil type in one group, respectively).

Moreover, increased phosphate fertiliser application could remobilise GLY because both compete for the same adsorption sites in soil. BORGGAARD & GIMSING (2008) and VEREECKEN (2005) showed that GLY, AMPA and phosphate have the same adsorption sites in soils, i.e. surfaces of iron and aluminium oxides, poorly ordered aluminium silicates and edges of layer silicates. BOTT et al. (2011) demonstrated the adverse effects of remobilised GLY on plants. In a greenhouse experiment, they analysed the growth of non-HR soybean in five different soils with regard to the above mentioned. Ten to 35 days before sowing, the GLY formulation Roundup UltraMAX® was applied at a recommended field application rate of 2-4 L/ha. Thereafter, soils were fertilised with different rates of phosphate (0-240 mg phosphate per kg soil). On soils without phosphate fertilisation, no phytotoxic effects of GLY were detectable. Conversely, phosphate fertilisation induced significant plant damage on GLY-treated soils. The findings of BOTT et al.

(2011) support the concept of rapid inactivation of GLY by adsorption to phosphate binding sites (e.g. SPRANKLE et al. 1975; GIESY 2000), mentioned in chapter 3.1.1, and its remobilisation through phosphate fertilisation. Adsorption sites in the soil for GLY and phosphate seem to be limited, and their absolute number likely depends on the soil type. Thus, AMPA may also be reactivated by phosphate fertilisation, but explicit studies are lacking.

As mentioned, increased phosphate fertilisation will enhance adverse effects on amphibians. Another supposed indirect effect of GLY could be eutrophication. Especially ephemeral ponds and small ponds with low buffer capacity, typical for amphibian breeding sites in agrarian landscapes, are at risk for eutrophication. AUSTIN et al. (1991) suggested that the degradation of GLY in water could increase the concentration of soluble phosphorus and consequently increase biomasses. Hence, the biodegradation of GLY in water could lead to eutrophication (AUSTIN 1991; SMITH 2003). However, compared to the intensive use of fertilisers today, this point seems to be of secondary importance.

Expansion of intensively used agricultural land and habitat fragmentation

HR crop systems were developed to facilitate weed management (one main reason of the HR technology), which leads to further aggregation of arable land as already happened in the USA where aerial application of herbicides is allowed (BENBROOK 2009, 2012). Furthermore, agriculture may expand in previously non-arable land as today new GM crops are developed, which can tolerate abiotic stress conditions (e.g. soil pH) (STEIN & RODRÍGUEZ-CEREZO 2009). It should be noted, that companies tend to combine (‘stack’) new traits, here the ability to withstand adverse abiotic conditions, with old traits, here GLY resistance. In Argentina, the national area of several anuran species meanwhile highly overlaps with the area of highest HR soybean cultivation (up to 89%: LAJMANOVICH et al. 2010) because agriculture expanded in previously uncultivated landscapes (PENGUE 2004).

Amphibian populations are often able to persist in agricultural landscapes via exchange within a meta-population framework (MANN et al. 2009). This means that, in theory, local (e.g. pesticide-induced) population declines, which transform such populations into ‘sinks’, may to some degree be compensated through ‘source’ populations (PULLIAM 1988). However, besides the species' biology (not all amphibians live in meta-populations: MARSH & TRENHAM 2001), especially the general landscape architecture determines, whether population are well linked and form a working and healthy meta-population. At least in the so called ‘developed’ countries, including Germany, the landscape is already highly fragmented and, therefore, most amphibian populations highly isolated too. This also leads to isolation with genetical and other drawbacks on amphibians as well as other taxa (see review: CUSHMAN 2006). Further expansion and aggregation of intensively used agricultural land will further fragment amphibian populations.

Conclusion

Potential direct and indirect effects of the HR technology on amphibians can be positive, but in most cases negative.

(i) Exclusive no-tillage farming leads to less soil erosion and, thereby, less contamination of nearby habitats. Temporarily uncultivated land is a suitable terrestrial habitat and resting place for amphibians. However, they are at risk, when farming activity resumes, e.g. when herbicides are applied.

(ii) According to some authors, intensive use of GBH has negative impact on soil, especially beneficial microorganisms. Nutrient lack and adverse effects on beneficial microorganisms (e.g. predators of plant pathogens) lead to increased fertiliser and pesticide use and, thereby, additionally affect amphibians.

(iii) With a view on other countries where GM crops are already cultivated for several years, an expansion and aggregation of intensively used agricultural land and, consequently, further loss and isolation of amphibian populations can be expected.

8. Discussion

8.1 Analytical approach

It is rather difficult to distinguish between long-term negative amphibian population trends due to anthropogenic factors and natural population fluctuations. Many factors (perhaps also unknown ones) and their interactions play a role in amphibian decline (e.g. COLLINS & STORFER 2003; GASCON et al. 2007). Therefore, the results of our statistical approach at maximum can be seen as correlative allusions, as we only considered factors related to agricultural practices. Other factors affect amphibian population sizes, for instance, the climate. Population dynamics were influenced through effects of climatic conditions on breeding activity and past recruitment (e.g. SEMLITSCH et al. 1996). However, precisely because the factors potentially affecting amphibian populations are perhaps ‘unmanageable’, we only considered agricultural variables in this approach. Hence, the results could only be related to the effects of the available agricultural variables on the considered amphibian populations. Nevertheless, first limited allusions on the influence of GBH applications could be made.

It is also very important how many populations were considered and for how long. For example, MEYER et al. (1998) analysed about 30 year long time-series of three Common frog populations and found large natural population fluctuations but no significant decline. These authors also considered aspects like predators and precipitation. Only one population showed a negative trend that could be explained by the introduction of goldfish, a heavy predator of Common frog tadpoles (GLANDT 1985). However, precipitation played no role in long-term fluctuations. Conversely, general negative population trends in amphibian species have been demonstrated in

Western Europe and North America using the ΔN method[85] (HOULAHAN et al. 2000; BONARDI et al. 2011). Amphibian populations declined and are declining worldwide, and in the USA and Western Europe the strongest declines occurred decades prior to the recently recognised ‘amphibian decline’ (HOULAHAN et al. 2000). These earlier declines could have been related to increasing industrialisation, especially of agriculture, in the so called ‘developed countries’. Therefore, for our analysis we have regarded as many populations and as long as possible from all parts of Germany and the USA. BONARDI et al. (2011) demonstrated strong recent declines of Common toad populations from Italy.

Unfortunately, detailed information on most abiotic and biotic factors potentially affecting populations was not available. Also, we had to be content with raw data on the annual consumption of agrochemicals of the whole country and not those of agricultural land near a certain breeding site or even of data from a certain German state. Naturally, allusions will be more precise the more detailed data are available. An assumption can exemplify this problem: concerning emerging trematode infections due to contamination with agrochemicals, BLAUSTEIN & JOHNSON (2003) stated that „…since the mid 1990s, however, at least 60 different species have been found to be affected in 46 U.S. states and parts of Canada, Japan and several European nations. The most severely affected areas include the western U.S., the Midwest and south eastern Canada“. The Western and Midwest of the USA are top areas of agricultural production, GM crops have been cultivated since the mid 1990s and today nearly all crops in these areas are genetically modified. Taking this fact, an indirect or direct causality between the trematode infections and the emerging cultivation of GM crops could be hypothesised. Now, if only the number of infections and the increase of GM crop production (or GLY applications) were considered, the results would probably show a strong correlation. However, if several other variables were taken into account, other predictors could explain more variance in the dataset and become more important. In general, a hypothesis cannot truly be tested as long as relevant detailed information on single populations and animals are lacking. Because the number of factors, which potentially affect amphibians is enormous and, therefore, largely lacking for single populations, scientist have to deal with this problem by conducting specific experiments in the laboratory, mesocosms or in the field (summarised in chapters 5.4 to 5.6 and discussed later) and by conducting monitoring in the field. Afterwards, the results from the specific experiments and the monitoring data have to be extrapolated to the ecosystem, which is mostly challenging as well.

Several authors already stated that *p*-values are a poor criterion for model selection (e.g. VAN BUSKIRK 2006; ELITH et al. 2008). More robust ‘modern’ techniques like the BRT model selection approach focus on regularisation by shrinkage instead. Taken together, BRT analysis allows an interpretation of single predictors in concert with their interactions. For our study this is more meaningful than ‘traditional’ correlation or regression models, mainly because we only

[85] The ΔN method “combines measurements of population change across multiple populations, and asks how large the `average' change in population size is over time” (HOULAHAN et al. 2000: 754-755).

regarded one (small) group of factors potentially affecting amphibians, i.e. agrochemicals and land use. Therefore, we see the BRT analysis as most meaningful method to test the data sets for correlations. Nevertheless, as with other statistical analyses, causative links between found correlations and real impacts on amphibians can only be suggested.

Amphibian populations from Germany

Although detailed data are lacking, the main allusion of our approach is that neither GLY consumption nor another agrochemical consumption/pesticide group stands out of the predictor sets used in the analysis of amphibian populations in Germany. Land use around breeding sites and in terrestrial habitats seems to be more important for the entirety of the populations. It has to be considered that (i) interactions with agrochemical consumptions influenced the results and (ii) contaminations at single sites can affect single populations. One could conclude that habitat modifications and destructions are more severe than any other factor in population declines in western countries, but as already mentioned, amphibian decline is a complex research field, a kind of 'puzzle' (BLAUSTEIN & WAKE 1995). Only by knowing the relevant factors affecting single populations, an overall conclusion can be stated. Because we are far from knowing all factors and interactions taking part in amphibian population decline, macroecological studies as ours could be a useful tool to sort different factors (here related to agriculture) and obtain a raw overview.

With regard to the results from Germany, in most cases, annual variation of predictors and their interactions mainly affected population dynamics. This is also not surprising because of the large, mainly natural fluctuations in amphibian populations. Furthermore, some land use variables seem to have either a positive or negative effect on growth rates of the considered amphibian populations. BRT calculations consider interactions between all variables. Hence, although the application rates of agrochemicals never explain a significant part of variance, explanatory land use variables differ when considering agrochemical applications one, three or four years prior. Forests are the preferred terrestrial habitats of the Common toad, but also ruderal areas can exhibit adequate habitat. These supposed positive impacts were also described by several authors (e.g. BLAB 1978; GÜNTHER & GEIGER 1996; KWET 2005; SOWIG & LAUFER 2007; AGASYAN et al. 2008). Conversely, urban areas and cultivated agricultural land offer several hazards for all life-stages like traffic (e.g. FAHRIG et al. 1995) or agricultural practices (e.g. DÜRR et al. 1999).

Results for Common frog populations give a mixed impression. Long-term negative population trends in Germany were observed especially in areas with intensive agriculture (SCHLÜPMANN & GÜNTHER 1996). Anyhow, the proportion of agricultural area in a 1 km buffer seems to have positive effects on growth rates. This can only be explained by the fact that after the major declines during intensification of the agriculture in Germany, Common frog populations in open areas are today principally stable in most German states (e.g. in North Rhine Westphalia; SCHLÜPMANN & GEIGER 2007). Hence, the observed interactions could show that today larger Common frog population can be found in open areas than in forested areas. This confirms other authors (e.g.

KWET 2005). However, up to now the populations have never reached the densities as in times before the industrialisation of agriculture after World War II (SCHLÜPMANN & GEIGER 2007). When the predictor set was considered three and four years prior respectively, proportions of urban areas apparently affected populations negatively. However, this cannot be related only to juvenile life-stages because threats in urban areas are principally the same for all life-stages of Common frogs, e.g. traffic (FAHRIG et al. 1995).

Adult individuals of the considered Moor frog populations seem to be positively affected by the presence of large parts of natural vegetation within agrarian landscapes. This is also described by, for instance, SOWIG (2007) who named extensive used structures between fields as main terrestrial habitats of amphibians inhabiting agrarian landscapes. Intensively used fields negatively affect larval and juvenile Moor frogs, probably due to harmful agrarian practices like pesticide applications or mechanical processing (cf. MANN et al. 2009). All conifer forests in the terrestrial habitat of the considered populations are plantations. The often monotone structures in such cultivations could explain the negative effect on Moor frogs.

Amphibian populations from the USA

For the set of summarised amphibian populations (1990-1998), the result has a very limited value due to both the limited data and the low model fit. The only conclusion that could be stated is that GLY consumption as a single predictor apparently has no explanatory power. This is not surprising as most population time series ended in 1996, the year of the adoption of GM crops, or before (see Appendix 1) and the pesticide consumption was considered at least one year prior. Hence, the rapid increase of GLY use was not included in this analysis (see Fig. 1). The annual variation in the whole predictor set seemed to be most important. Furthermore, an overall negative impact of the total use of fungicides and bactericides could be hypothesised for juvenile life-stages of North American amphibians in the years 1990 to 1998, but this is rather speculative. However, acute toxicity, genotoxic and teratogenic effects of fungicides and bactericides at environmentally relevant concentrations on amphibian larvae have been demonstrated (e.g. VENEGAS et al. 1993, BELDEN et al. 2010).

With regard to call surveys (2001-2010), the final models had a very low to low fit respectively, limiting their information. The annual variation in the whole predictor set explained the largest part of variance for both species. Total use of fertilisers seemed to have slightly negative effects on juvenile life-stages of Northern cricket frogs and adult American toads, whereas total use of herbicides apparently affected juvenile life-stages of American toads negatively. Negative impacts of fertilisers on different amphibian life-stages are summarised by MANN et al. (2009) and in chapter 5.6.2.1. Several studies concluded significantly negative impacts of herbicide treatments including mixtures on larval American toads (e.g. RELYEA 2004, 2009; BRODMAN et al. 2010; WILLIAMS & SEMLITSCH 2010). Hence, an overall negative affect may be plausible. In the same way, larval Northern leopard frogs were suggested to be at risk due to herbicide applications (e.g.

CHEN et al. 2004; HOWE et al. 2004; RELYEA 2005c; BRODMAN et al. 2010). For Northern leopard frogs, it was the only case that GLY applications, together with metolachlor-S, stood out of the data set. As a consequence, also a considerably negative impact of both herbicides on adults could be postulated. This could be related to direct over-spraying of adults, which, for instance, have to cross fields during migration (e.g. RELYEA 2005b; BERNAL et al. 2009b; DINEHART et al. 2009), but this is only a hypothesis.

Conclusion for the macroecological approach

Taken together, the variation of all considered predictors mainly explained variance in most cases. Hence, single important predictors were few and exhibited only low explanatory power for most amphibian populations from both regions. In Germany, land use surrounding populations apparently has a higher impact on population growth rates than certain agrochemicals. In the USA, we only considered land use change from conventional to GM crops and total agricultural area of the whole country due to lack of data. None of these predictors stood out of the data set. Limited data availability could explain the very low model fit for the 'old' North American amphibian populations (1990-1998) and most of the more recently considered populations (2001-2010). Nevertheless, results indicated negative impacts of fertilisers and herbicides and, in the case of adult Northern leopard frogs, also for GLY. However, as already mentioned several times, these results could only be seen as correlative allusions. As a result of our analytical approach, some relevant questions emerged which cannot be answered without the help of laboratory tests, long-term field studies and monitoring:

(i) Are amphibian populations in Germany, especially in agrarian landscapes, more threatened by land use and changes in their habitats than due to agrochemical consumptions (including GLY applications in no-tillage farming)?

(ii) Did the total use of fungicides and bactericides in times before the adoption of GM crops really have an important negative effect on North American amphibian populations?

(iii) Do supposed important predictors (i.e. total use of fertilisers and herbicides) actually act species- and life-stage-specific?

(iv) Are wild adult Northern leopard frogs more sensitive to metolachlor-S and GLY applications than other species?

8.2 Literature review

We reviewed around 50 available studies on impacts of GLY and GBH on amphibians including laboratory, mesocosm and field studies. Most of them have been conducted in North America. No single study deals with amphibian species, which are distributed in Germany. Summarised, different authors came to different conclusions concerning the impact of GLY use on amphibians. It has to be accentuated that often the same working groups and scientists either concluded negative impacts of GBH under real-world scenarios or not (cf. the references), probably due to different view points on pesticide use in general. In our opinion, to date no general conclusions with regard to impacts of pesticide use (including GBH use) on global amphibian decline can be drawn.

Some years ago, the most recognised study ‘The lethal impact of Roundup on aquatic and terrestrial amphibians’ was published in the journal ‘Ecological Applications’ by R.A. RELYEA (2005b) and prompted Monsanto Company (many HR crops are patented by this company and until 2000 also GLY was patented by Monsanto in the USA) to give a statement[86]. In addition, the publication caused THOMPSON et al. (2006) – who concluded no adverse effects of aerial GBH applications on amphibians in their studies – to write a letter to the editor of ‘Ecological Applications’. Like Monsanto, they denounced the experimental design by RELYEA (2005b), in particular the tested concentrations. Application rates would be atypical of those commonly employed in agriculture, forestry and industrial use and tested GLY concentrations would exceed concentrations typically observed in natural waters. Furthermore, the tested GBH is only labelled for private use and a direct over-spraying of water surfaces would be an unlikely scenario. The dramatic effect of induced mortality, as seen by RELYEA, would simply reflect the high concentrations used. Furthermore, THOMPSON et al. (2006) complained about not citing their papers. In his reply, RELYEA (2006) provided “evidence to demonstrate (i) that application rates under real-world conditions are wider ranging than the authors suggest, (ii) that environmental concentrations cited by THOMPSON et al. (2006) are a highly biased subset of existing data, (iii) that the suspected flaws reflect a lack of knowledge of aquatic ecology, and (iv) that previous risk assessments are largely irrelevant to assessing Roundup®'s risk to tadpoles.” Relyea’s study was submitted seven months prior to the publication of their works. Therefore, he was not able to cite the papers of THOMPSON and colleagues. Furthermore, although Roundup® products must not be applied on water surfaces, it has been found in aquatic habitats by several scientist including THOMPSON and colleagues. The motivation of THOMPSON et al. (2004) for their study on Vision® and amphibian larvae was that small ponds are not protected by no-spray buffer zones and they are difficult to avoid during aerial application. Hence, they had shared the view of RELYEA to examine the impacts of this factual circumstance. In addition, RELYEA (2006) stated that THOMPSON et al. (2006) had only considered very low environmental GLY concentrations (0.005 to 0.55 mg a.e./L) and dismissed the higher ones in surface waters, which were also published, and estimated worst-case-scenarios (see chapter 5.1). THOMPSON et al. (2006) really provided a biased perspective because, for instance, they only cited the low GLY concentrations 49 days after application (<0.005 mg a.e./L), but not the high ones (0.3-0.7 mg a.e./L) found on the day after treatment by WOOD (2001). Furthermore, considered data were sometimes from rivers, which are typically lower in pesticide concentrations due to flushing action. In addition, most North

[86] Monsanto Company stated that the conclusions by RELYEA (2005b) would be misleading because non-realistic concentrations were used and applied directly over water against the product instructions. The internet statement of Monsanto Company is not longer available, but a short (German) summary can be seen at http://www.karch.ch/karch/d/ath/roundup/roundfs2.html.

American tadpoles do not live in streams. Altogether, RELYEA (2006) stated that "we should come to terms with the unfortunate fact that there are just very few data on how much Roundup appears in tadpole habitats."

This data gap has several causes. Data on GLY in environmental samples are often limited because analysis is expensive and logistically difficult (see chapter 5.1). Furthermore, FREUZE et al. (2007) showed that a commonly used preparation step in the laboratory, the FMOC-Cl (= fluorenylmethyloxycarbonyl chloride) derivatization, can sometimes lead to an underestimation of GLY and AMPA in water. However, the main problem when determing environmental concentrations of GLY seems to be its different fate in different soils and waters (see chapter 3.1): Differing half-lives make a comparison of environmental measurements very difficult. Several studies have shown that acute toxicity occurs mainly within the first 1-3 days (e.g. RELYEA 2005b,c; RELYEA & JONES 2009). Hence, the highest peaks in breeding ponds are of special interest. Due to the often rapid breakdown of GLY, it is reasonable to assume that the few so far measured environmental concentrations are often too small and do not represent peak concentrations because in nearly all cases sampling did not occur directly after application (direct over-spraying or drift) or after the first heavy rainfalls (run-off). GLY concentrations in run-off water could be correlated with precipitation rates in the experimental field studies by HENKELMANN (2001) and the environmental findings by PERUZZO et al. (2008).

Keeping the classic toxicology maxim at the back of our minds ("The dose makes the poison!"), real-world concentrations have to be taken in consideration when calculating toxicological endpoints. For example, in Europe concentrations of pesticides in surface waters are calculated using so called FOCUS scenarios (for further information see DANIEL et al. 2007). Another problem in ecotoxicological risk assessments that could be solved is the use of proxy organisms (here fish for amphibians). With regard to acute toxic effects (mortality), larval fish and aquatic invertebrates can be accepted as proxy organisms for tadpoles, provided regular safety factors are taken into account, as figured out in chapter 5.4 and also stated by other authors (e.g. ALDRICH 2009). However, risks for terrestrial life-stages seem to be higher for amphibians with their thin, permeable skin as for proxy organisms (other vertebrates) (QUARANTA et al. 2009; RELYEA 2011) and for chronic and delayed effects standardized 'amphibian-specific' endpoints should be considered. Hence, amphibians cannot be entirely replaced by proxy organisms and should be included in risk assessments.

However, one general problem of ecotoxicological risk assessment is the extrapolation from the laboratory to the field. Here, mesocosm or field experiments should help, but it is very difficult, labour and time intensive to consider all factors taking part in a real-world aquatic community.

The main question is if GLY and GBH use affects natural amphibian populations. Herbicide use is one among many supposed reasons causing on-going global amphibian decline and extinction (BOONE et al. 2007). However, its exact role remains difficult to assess as (i) field data remain sparse and (ii) abnormal population changes have been suggested to often result from multiple interacting causes (e.g. STUART et al. 2004, 2008). Against a main role of GBH use on amphibian decline speaks that the majority of declines in developed countries occurred in the 1950s to 1960s (HOULAHAN et al. 2000) and GLY marketing only started in 1974 (DILL et al. 2010). Furthermore, recent declines and extinctions of amphibians have been witnessed over the last three decades in pristine and remote areas in the tropics (MENDELSON et al. 2006). Although the findings of KAISER et al. (2011) show minimal pesticide drift into such areas, GBH use as reason can be ruled out here because other potential causes have been identified like habitat destruction and emerging infectious diseases (STUART et al. 2008). As figured out in chapter 5.4, several studies reported adverse effects of some GBH (especially those with tallowamine surfactants like POEA) on amphibians at concentrations that can occur in the environment (cf. chapter 5.2), but also that other less harmful GBH (e.g. that are labelled for aquatic use) will probably not affect natural amphibian populations in normal-use scenarios. For example, the highest observed GLY concentrations are 0.7 mg a.e./L (directly found in amphibian habitat) and 1.95 mg a.e./L after a realistic aerial application at approved rates. With regard to worst-case EEC, some scientists calculated relatively high concentrations if shallow water bodies would be directly over-sprayed (7.6 mg a.e./L). Worst-case EEC for Germany (agricultural application at maximum approved rates next to a shallow water body and without buffer strip) is 0.9 mg a.e./L. For some GBH, all these concentrations are similar or even higher than the LC50 values for tadpoles and would have adverse effects on tadpole survival. Conversely, other GBH would have no effects on tadpoles at these concentrations. Hence, the formulation used and the site-specific application practice make the difference. For example, aerial applications are not allowed in Germany but aerial applications of Vision® (= Roundup Original®) are conducted in Canadian forest management. Another main problem in assessing the risk of GBH applications for wild amphibian populations is that – even with co-stressors – tests considered mostly tadpoles and acute toxicity rather than other life-stages or parameters. It is important to note that the majority of anuran species follow a reproductive strategy of 'overproduction' enduring high mortality rates of their offspring, and, therefore, (subjectively) high mortality rates in larvae may not substantially affect population viability (SCHMIDT 2004b). Preliminary results of a population viability analysis for several Green toad populations, that is based on acute toxicity and tadpole survival, suggest that only unrealistic concentrations of GBH (about 40 mg a.e./L) would lead to population declines (WAGNER et al. 2012). However, this model only considered average toxicity of several GBH and did not consider chronic and delayed effects (e.g. decreased over-winter survival of metamorphs after exposure to sublethal concentrations) and impacts on terrestrial life-stages were not considered. Including these may change the results. Finally, there are practically no data on amphibian populations within agrarian landscapes, e.g. on population sizes or habitat connectivities. Data on these parameters would be also required for population viability analysis on both local and larger scales.

Some years ago the USEPA already assessed the risk of GLY use to the Californian red-legged frog (*Rana draytonii*) and recommended some management measures (CAREY et al. 2008). Potential exposure of GLY and its formulations to the aquatic phase of *R. draytonii* was assessed using simple dilution calculations based on the mass of the applied herbicide and the volume of the water body and supplemented with data from the NAWQA program (see also chapter 5.1). Estimated worst-case concentrations were very low compared to other EEC (0.21 mg a.e./L). Potential exposure of the terrestrial habitats and the prey with GLY was modelled using three different models of the USEPA. However, the conclusion of this assessment was limited because potential risks to aquatic habitat and prey have been partly deduced from ecotoxicological endpoints of fish and small mammals. Although CAREY et al. (2008) determined a 'May Affect' and 'Likely to Adversely Affect' for *R. draytonii* from the use of GLY. Additionally, they determined that there is the potential for modification of designated critical habitat from the use of the chemical. However, not the agricultural but forestry applications and applications on impervious sites like highways are considered as potential dangers. Nevertheless, one formulation (MON-14420) was assessed to potentially endanger frogs in their terrestrial habitat due to high acute toxicity values. Taken together, this report was the first risk assessment of an agency on the potential risk of GLY use on a threatened amphibian species, but it only provides limited allusions.

In general, the impacts of a certain agrochemical like a GLY formulation, but also of other agrarian practices on certain amphibian populations can definitely only be evaluated species and site-specific. The report by CAREY et al. (2008) can be seen as a first approach, we tried to improve, but – as already mentioned – more data are urgently needed. However, because a detailed evaluation is not possible in most cases, the ecotoxicological risk assessments on the impact of pesticides in general have to be improved too, especially concerning native amphibians.

Legal basis for the improvement of amphibian monitoring and conservation in the agrarian landscape

As already mentioned in the introduction, the Directive 2001/18/EC of the European Parliament and the Council requires a risk assessment of GMO including direct, indirect, immediate and delayed effects on the environment[87], before a GMO can be authorized. The Directive also requires implementing a monitoring plan to trace and identify any direct, indirect, delayed or unforeseen effects of GMO after their placing on the market. That naturally has to include amphibians. In general, the precautionary principle should be considered. The European Directive 2004/35/EC on environmental liability with regard to the prevention and remedying of environmental damage[88], in Germany regulated by the 'Umweltschadensgesetz'[89] have to be considered when environmental damages due to agricultural practices occur. It has to be repeatedly highlighted that effects impacting amphibian health and destruction of amphibian

[87] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:106:0001:0038:EN:PDF
[88] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:143:0056:0075:EN:PDF
[89] http://www.gesetze-im-internet.de/bundesrecht/uschadg/gesamt.pdf

habitats due to agricultural practices would be a violation of national law, especially a violation of § 5 of the German 'Bundesnaturschutzgesetz'[90] as well as of § 6 of the German 'Pflanzenschutzgesetz'[91]. In some cases, i.e. when a population of a strictly protected species (annexe IV of the European Habitats Directive[92] and, therefore, also strictly protected in Germany) seems to be affected by agricultural practices, it seems to be inevitable to conduct a species and site-specific evaluation by experts (Art. 12 of the European Habitats Directive[93], in Germany regulated in the BNatSchG). One goal of the National Biodiversity Strategy of Germany[94] is that by 2015, the proportion of land use for valuable conservation agro-biotopes should increase by at least 10% compared to 2005. Likewise the European Council regulation No 73/2009[95], which establishes common rules for direct support schemes under the Common Agricultural Policy of the EU, states that EU subsidies for farmers have to be coupled to activities in environmental protection (so called 'Cross Compliance'). Taken together, the legal basis for effective amphibian conservation in 'modern' agricultural areas exists.

8.3 Conclusion

In our opinion, to date no general conclusions with regard to impacts of pesticide use (including GBH use) on global amphibian decline can be made. Hence, only species- and site-specific evaluations can be conducted. We see the need to improve population viability analysis for wild amphibian risk assessment. Therefore, field data of both amphibian populations in the agrarian landscape and contamination with GBH and other pesticides are needed, but these are widely lacking.

In the introduction of this expert opinion, we formulated some key questions. Following answers can be given:

- *Which concentrations of GLY and its main metabolite can be found in the environment?*

GLY and AMPA monitoring data are sparse, mainly because analysis is expensive. Only data on aquatic habitats are available. Maximum GLY concentrations, which have been found in the environment are 0.7 mg a.e./L in small water bodies next to HR soybean cultivations in Argentina and 1.95 mg a.e./L in a forest pond after aerial applications in Canada. Worst-case EEC for surface waters from national agencies are 1.44 mg a.e./L for Canada (where aerial applications are approved) and 0.9 mg a.e./L for Germany (application without buffer strip). Some scientists

[90] http://www.gesetze-im-internet.de/bundesrecht/bnatschg_2009/gesamt.pdf
[91] http://www.gesetze-im-internet.de/bundesrecht/pflschg_1986/gesamt.pdf
[92] For strictly protected German amphibian species see http://www.bfn.de/fileadmin/MDB/documents/themen/natura2000/artenliste.pdf
[93] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1992L0043:20070101:EN:PDF
[94] https://secure.bmu.de/fileadmin/bmu-import/files/english/pdf/application/pdf/broschuere_biolog_vielfalt_strategie_en_bf.pdf
[95] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:030:0016:0099:EN:PDF

calculated higher EEC up to 7.6 mg a.e./L for direct over-spraying of shallow water bodies. AMPA has been found at lower concentrations, but at higher frequencies. No EEC for AMPA is available.

The main conclusions of these results are that both GLY and AMPA are present in environmental samples of the Americas and Europe, but usually at low levels. However, maximum GLY concentrations in the environment may be higher than those measured, because sampling usually did not take place directly after application or first heavy rainfalls after application. Maximum GLY concentrations are of main interest because toxic effects of GBH on tadpoles mainly occur within the first 24h. The published worst-case scenarios seem to represent good estimates and, apparently, can be used for risk assessment of amphibians. For Germany, the worst-case EEC of 0.9 mg a.e./L (calculated by the BVL 2010c) should considered. Nevertheless, (i) detailed information on real contamination levels of aquatic and terrestrial amphibian habitats is widely lacking, (ii) amphibian toxicity studies on AMPA are lacking and (iii) the main problem remains that measured GLY concentrations do not identify the used GBH, but surfactants are mainly responsible for adverse effects.

- *What are possible exposure pathways to different amphibian life-stages?*

Potential exposure pathways are numerous and mainly include (i) direct over-spraying of migrating and resting terrestrial life-stages, (ii) contact with contaminated plant material and soil, (iii) contamination of breeding ponds with acute, chronic and delayed effects on aquatic life-stages and (iv) ingestion of contaminated food or sediment (for details see chapter 6).

- *Does the agricultural change in the Americas and the resulting increased use of GLY correspond to amphibian populations decline?*

The statistical approach cannot answer this question because of its limitations. In one case, correlative allusions hypotheses an impact, but information are very limited due to limited data availability and low model fit. No causative link between increased GLY use and declining amphibian populations in the Americas can be stated.

- *Are there yet any 'signs' for negative impacts on amphibian populations in Germany as a result of an increased deployment of GLY in the conventional agriculture?*

Here, the statistical approach hypotheses no impacts on amphibians by GLY use in Germany up to now. While no-tillage farming in Germany is still relatively rare (e.g. compared to the Americas), its impacts on amphibians have to be compared with traditional methods. At a rough estimate, both methods (ploughing and non-selective herbicide applications) have the potential to seriously harm individuals, but their potential to affect amphibian populations, especially nationwide, remains unclear.

- *What do we actually know about the impacts on amphibians of GLY and its* *formulations?*

Direct reported acute effects on amphibians include – besides increased mortality – damages of the gills of tadpoles and different malformations, inhibition of vital enzymes and release of oxidative stress; chronic and delayed effects include supposed endocrine disruption, delayed but also precipitated time to metamorphosis and reduced fitness at hatching, which later on can increase mortality (see chapters 5.4 and 5.5 for detailed information). Sometimes, effects were demonstrated at environmentally relevant concentrations. Most likely, added substances (surfactants) rather than GLY itself are responsible for adverse effects and species-specific responses have been observed. Hence, some GBH can be regarded as toxic at least for some amphibian species. As already mentioned, adverse impacts of an increasing GBH use due to a possible introduction of HR crops on amphibian populations and communities can only be postulated for worst-case scenarios, mainly because of lacking data but also due to species, life-stage, formulation and application-specific (i.e. cultivation-specific) effects. The effects of long-term and regular applications of GBH on wild amphibians should be monitored.

- *Can there be identified (potential) effects on amphibian species or populations as a result of the use of GLY coupled with biotic or abiotic stressors?*

Several authors found different interactions of GBH and other stressors (see chapter 5.6). In most cases, either another stressor increased the toxicity of the herbicides or the formulations increased the effect of the other stressor. Amphibian populations in anthropogenically influenced landscapes are adversely affected by different stressors and, therefore, herbicide applications should not be regarded separately.

- *Are amphibians differently affected by the cultivation of conventional crops compared with HR crops especially with respect to the different weed management systems?*

Conclusions concerning this question have to be hypothetical because of the limited data available for the Americas. Furthermore, the risk of no-tillage farming compared to traditional tillage methods can only be postulated. Nevertheless, the commercial HR cropping system with its complementary non-selective herbicide, often GBH, allures to skip crop rotations. Hence, weed resistances and subsequent equal or even higher herbicide applications are likely. However, if GBH are more dangerous to amphibians than the selective herbicides which were replaced, remains unknown. In general, GM crops facilitate the expansion of agriculture in less profitable land (further habitat destruction) and the aggregation of fields (further isolation and habitat destruction). An intensive meta-analysis, perhaps including extra laboratory studies and modelling of the environmental fate, has to be conducted.

- *What kind of data is missing with regard to obtaining a more conclusive picture of effects of GLY to amphibians?*

The following chapter 9 (‘analysis of deficits’) enters into this question.

9. Analysis of deficits

Our macroecological approach and the literature and database review have revealed a magnitude of missing data necessary to respond comprehensively to the original question of this expert opinion on the possible correlation of the worldwide amphibian decline and the increasing use of GLY in the agrarian industry. Although we found some data on concentrations of GLY and its main metabolite AMPA in environmental samples, more information on average and maximum concentrations in the environment are needed in order to assess potential risks for wild amphibian populations, especially for (i) breeding ponds (permanent but especially small and ephemeral), (ii) run-off from fields, and (iii) herbicide drift affecting amphibians in both breeding ponds and terrestrial habitats. When GLY concentrations are measured in breeding ponds, the temperate water stratification observed by RELEYA (2009) and JONES et al. (2011) should be considered, because concentrations near the water surface are especially important. Furthermore, pesticide monitoring should be timed with herbicide applications, taking into account consecutive heavy rainfalls so that highest peak concentrations can be captured. These are important because of the relatively rapid dissipation of GLY and because acute toxic effects on tadpoles have been often observed within the first 24h of GLY exposure (e.g. RELYEA 2005b). Finally, surfactants are mainly responsible for adverse effects and need to be included in pesticide monitoring.

The analysis of the experimental studies showed that there are no studies on the impact of AMPA on amphibians. Therefore, it cannot be assed whether the environmental concentrations of AMPA are critical or not. In this context, the observed persistence and long-term leaching of the metabolite is mentionable.

With regard to different GBH and their impacts on amphibians, several further studies have to be conducted. Here, a chain of study types seems to be relevant (i.e. laboratory experiments followed by field and mesocosm studies), because specific differences have been observed for amphibian species, life-stages, formulations and crop cultivations.

Below, some more examples of additional studies of potential interest are listed:

(i) TRUMBO (2005) tested the toxicity of GLY isopropylamine salt alone and a nonylphenol ethoxylate-based surfactant (NEP), each separately, on aquatic organisms (two fish and a crustacean species) other than amphibian larvae. When the two compounds were tested together in a 2:1 mixture, the toxicity of the surfactant changed little, but the toxicity of glyphosate changed dramatically. The cooperation between GLY and NEP should be investigated with tadpoles, too. In general, the toxicity of other surfactants than POEA should be evaluated. However, for this purpose surfactants of GBH have to be known (as already demanded by other authors; e.g. COX & SURGAN 2006; LAJMANOVICH et al. 2011).

(ii) Because nonylphenol is known as an endocrine disruptor in fish and other vertebrates, its effects on amphibians should be investigated as well. Furthermore, it has to be identified if GBH (also with other surfactants) can affect the sex hormone balance in amphibians and possibly impact the sex ratio within a population.

(iii) Currently, immunosuppressive effects of GBH can only be supposed. There is only one study that supposes immunosuppression of GBH (elevated trematode infection: ROHR et al. 2008a), but no study supposes immunosuppressive effects of GBH regarding chytridiomycosis (e.g. GAHL et al. 2011).

(iv) TAKAHASHI (2007) showed that treefrog females are potentially able to avoid ponds with predators and/or herbicide contamination. However, his study design was limited (only four ponds) and he only used one high herbicide concentration. Conversely, no effects of environmentally relevant concentrations of AMPA, GLY and a GBH on site selection (not oviposition site selection) by three European amphibian species could be observed (WAGNER & LÖTTERS 2013). Hence, it seems important to test if predator cues together with several low, environmentally relevant concentrations have an impact on female amphibians when they select their oviposition site. Furthermore, the biological mechanism why predator cues lead to higher mortality rates in tadpoles is still unknown.

(v) Only ORTIZ-SANTALIESTRA et al. (2011) conducted a study on interactions between a GBH and a fertiliser. Because both compounds are frequently applied and often occur simultaneously in agrarian landscapes, further research is strongly recommended. This also applies to studies on impacts of pesticide mixtures. Furthermore, it should be investigated at the molecular level, why exposing amphibian embryos to GBH and fertilizer increased their body length at hatching and what the ecological consequences might be.

(vi) There are no studies on the impacts of agriculture on amphibians that also take into account climate change. We are unaware of studies on interactions between any extreme weather conditions (changing temperatures, earlier pond drying, shifts in application time in no-tillage and GM crop farming etc.) and GLY use.

(vii) Last but not least, potential indirect effects along the food chain should be considered. So far it has not been studied what impacts insects and arthropods as feed have on amphibians, when they get contaminated by GBH (or other pesticides) on the acre and then enter nearby amphibian habitats which are supposed to be ‘unaffected by agriculture’.

Nearly no data on native European amphibian species are available. Studies showed species-specific differences in sensitivity, also concerning the GBH formulation. Which (tadpoles of) native amphibian species in Germany are currently the most sensitive or the most tolerant ones can only be assumed (e.g. that European treefrogs could be more sensitive and toad species more resistant), because most available LC50 values cannot be directly compared due to differences in the study designs and provide a rough estimate only. Without standardization mass animal testing is neither useful nor necessary. Furthermore, different authors came to different conclusions about how the results obtained from laboratory studies apply to field conditions. For example, while DINEHART et al. (2009) conclude that toxicity of GBH will decrease in the field due to the presence of moistened soil or leaf litter, RELYA (2005b) denies this. For risk assessment it seems to be more meaningful at present to consider what can be concluded at least and what at most from available studies rather than to perform further animal testing.

Data on amphibian populations in agrarian areas is limited for North and especially South America, but also Germany. A random amphibian survey near GM crop cultivations in the Americas shall be conducted. Although there are outstanding long-term studies on the population dynamic and migration behaviour of amphibians in agrarian areas in Germany (see KNEITZ 1998, HACHTEL et al. 2006; BERGER et al. 2011), a nationwide, at least random, survey on amphibians in and nearby different crop cultivations is still lacking. Surveys would have to include literature and database (e.g. from nature conservation authorities) information, accompanied by field observations and real-world data on GLY, AMPA and especially surfactants, e.g. when maize is sown and most amphibians in Germany breed (cf. chapter 6.1).

No-tillage farming is often declared as more ecological and beneficial for agrarian biodiversity (e.g. WARBURTON & KLIMSTRA 1984). One further question that arises is if the application of non-selective herbicides during no-tillage farming has similar, less or more impacts on amphibians in the field than alternative tillage like ploughing etc. (for a comparison of different tillage methods concerning amphibian health see BERGER et al. 2011). On first sight, it seems to be of equally negative impact if migrating or resting amphibians encounter ploughing or over-spraying with non-selective herbicides (perhaps more individuals would even survive the herbicide applications). However, some amphibians perhaps prefer no-tillage fields as a terrestrial habitat because plant residues etc. offer more hiding places. Hence, more individuals would be attracted in late summer and early autumn to no-tillage fields and encounter over-spraying with non-selective herbicides, for instance, in no-tillage farming for winter grain cultivation. To answer this question, specific studies on migration behaviour (e.g. radiotelemetry) in no-tillage fields should be conducted.

10. Acknowledgements

First, we would like to thank especially B.R. SCHMIDT (Koordinationsstelle für Amphibien- und Reptilienschutz der Schweiz, KARCH) for offering data and for valuable comments. Furthermore, we thankworthy obtained permission to use count data from drift fences collected by volunteers. Due to several restricting reasons, figured out in chapter 4.2, we did not use all available data at http://www.amphibienschutz.de/. Following responsible persons have given us permission to use data for analysis (in alphabetical order): C. AHRENS, R. APPL, R. ARAMAYO, T. BEUSTER, P. BLOCK, M. BOTHE, M. BUCK, G. EBERIUS, B. EFKEN, A. ELSER, M. DIETRICH, R. DUNKER, W.-R. GROSSE, R. GRUBE, M. HABERMANN, M. HACHTEL, H. HAGENBERG, G. HOFFMANN, J. HUTFLUSS, I. JÄDTKE, B. KITZMANN, L. KOCH, J. KUNZ, S. KÖNIGSMARK, M. KRAUS, H. LENK, G. LINDNER, H. LÜHMANN, C. LYNEN, M. MASCHEWSKI, R. MEIER, U. MILLER, I. MÜHLBERGER, G. NINTZEL, L. OSTER, R. PETER, W. PETERS, R. PIETSCH, E. POSTLER, W. POSTLER, F. PRASSE, R. PRIOR, H. RABENECK, J. REINECKE, J. ROZYCKI, C. RUHRMANN, R. RUMMEL, J. SCHARON, I. SCHERBER, S. SCHLEICH, C. SCHMIDT, H. SCHMITT, T. SCHÖNBRODT, H. SCHÖTTLER, S. SCHREINER, B. SCHRÖDER, K. SEIDER, J. SEIFERT, D. SIEBENEICHER, K. SOLBACH, A. SZYMANSKI, F. TODT, G. VITZTHUM, I. VOLLMER, R. WAGENSTALLER, J. WALCZYNSKI, G.D. WALTER, H.J. WINDELN. It must be mentioned that drift fences were usually attended by several volunteers, often organised in regional groups of environmental and conservation organisations (in particular of the NABU and the BUND, Friends of the Earth Germany). We would like to thank all the helpers without them many amphibian populations would have been disappeared due to road traffic. Let's hope that some day Germany will attend to its duty and will not only provide funds for assembly and dismantling of drift fences, but also for the manpower at night.

Last but not least, we want like to thank P. HORCHLER for his valuable help with the BRT, H. TEICHMANN and especially W. REICHENBECHER for valuable comments on the manuscript.

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12. Appendices

Appendix 1: Species of the database by HOULAHAN et al. (2000) used in analysis

Order	Family	Species	No. of populations	Study duration
Caudata	Ambystomatidae	Californian tiger salamander (*Ambystoma californiense*)	1	1992-1994
		Jefferson salamander (*Ambystoma jeffersonianum*)	1	1991-1993
		Blue-spotted salamander (*Ambystoma laterale*)	1	1991-1995
		Spotted salamander (*Ambystoma maculatum*)	4	1991-1997
		Marbeled salamander (*Ambystoma opacum*)	2	1991-1997
		Mole salamander (*Ambystoma talpoideum*)	1	1991-1994
		Pacific giant salamander (*Dicamptodon tenebrosus*)	1	1992-1994
	Plethodontidae	Sacramento mountain salamander (*Aneides hardii*)	5	1992-1996

		Dusty salamander (*Desmognathus fuscus*)	1	1993-1996
		Seal salamander (*Desmognathus monticola*)	1	1991-1993
		Northern two-lined salamander (*Eurycea bislineata*)	1	1994-1997
		Eastern newt (*Notophthalmus viridescens*)	7	1991-1995
		Redback salamander (*Plethodon cinereus*)	2	1991-1996
Anura	Bufonidae	American toad (*Anaxyrus americanus*)	2	1991-1996
		Boreal toad (*Anaxyrus boreas*)	2	1991-1994
		Houston toad (*Anaxyrus houstonensis*)	1	1991-1998
		Southern toad (*Anaxyrus terrestris*)	1	1991-1994
	Hylidae	Spring peeper (*Pseudacris crucifer*)	2	1991-1996
	Microhylidae	Eastern narrowmouth toad (*Gastrophryne carolinensis*)	1	1991-1994
	Leiopelmatidae	Coastal tailed frog (*Ascaphus truei*)	4	1992-1994
	Ranidae	Green frog	5	1991-1996

	(*Lithobates clamitans*)		
	Relict leopard frog (*Lithobates onca*)	2	1991-1996
	Pickerel frog (*Lithobates palustris*)	1	1993-1996
	Northern leopard frog (*Lithobates pipiens*)	3	1991-1996
	Wood frog (*Lithobates sylvaticus*)	2	1991-1996
	Foothill yellow-legged frog (*Rana boylii*)	1	1992-1996
	Oregon spotted frog (*Rana pretiosa*)	1	1995-1997