Bundesamt für
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Glyphosate - Comments from Germany on the paper by Paganelli, A. *et al.* (2010): „Glyphosate-based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signaling“

Scope

The scientific article ”Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signalling” by A. Paganelli, V. Gnazzo, H. Acosta, S.L. Lopez and A. E. Carrasco has been electronically published in “Chem. Res. Toxicol.” on August 9, 2010.

The focus of the first part of the following evaluation is on the question whether the presented results can be used for or may have an impact on the risk assessment of plant protection products containing glyphosate as active substance for human health.

Effects on amphibians and the relevance of the described findings and the appropriateness of the conclusions drawn from the experimental results in relation to possible environmental effects are assessed in the second part.

1. Impact on risk assessment with regard to human health

Results

Under highly artificial exposure conditions (co-cultivation or microinjection), glyphosate and the glyphosate-based herbicide *Roundup Classic* may cause serious developmental effects in African clawed frog (*Xaenopus laevis*) and chicken embryos with the neural crest being the primary target. The mode of action might be similar to that one of excess vitamin A / retinoic acid.

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Even though basic mechanisms of development and its disturbances can be studied in such models, it is hardly possible to predict adverse effects of certain substances on mammals on this basis. In particular, exposure conditions are extremely different and highly artificial.
There is a huge and reliable database for developmental toxicity of glyphosate and no evidence of teratogenicity has been obtained. In particular, studies in rats and rabbits failed to reveal craniofacial malformations as they would be expected if a substance affects mainly the neural crest. Furthermore, there is no epidemiological evidence in humans that glyphosate (herbicides) might be teratogenic. Despite to what is claimed by the authors, there is no clear-cut link to a hypothetic increase in malformations in regions with extensive use of plant protection products in South America.

Thus, the findings do not put the current risk assessment for glyphosate and glyphosate-based PPP into question with regard to human health.

Reasons

The authors investigated possible effects of glyphosate and a commercial formulation (*Roundup Classic* containing 480 g/L of a glyphosate salt, presumably the isopropyl ammonium salt) on the development of African clawed frog (*Xaenopus laevis*) embryos. This amphibian model is widely used in embryology and developmental physiology. Frog embryos were cultivated in the presence of three different concentrations of the glyphosat-based herbicide. In another experiment, the active ingredient itself was directly injected into frog embryos.
It could be demonstrated that glyphosate as well as its herbicidal formulation may disturb early stages of frog embryo development by interfering with key molecular mechanisms. There were serious effects on neural crest resulting in alterations of segmentation, lateralisation, neural and skeletal (cartilage) structures. Eye and brain development were also affected. This variety of effects is not surprising because neural crest cells differentiate into various tissues.
These findings resembled teratogenic effects that occur in vertebrates either in maternal deficiency of vitamin A or in excess of retinoic acid. Indeed, in further experiments, some evidence was obtained that retinoic acid activity was increased in embryos after exposure to glyphosate and/or Roundup.

Furthermore, chicken embryos were incubated with Roundup after injection of 1:3500 or 1:4500 dilutions into the eggs. Similar effects on embryo development as in *Xaenopus laevis* were observed resulting in the reduction of optic vesicles and evidence of microcephaly.

It is known that abnormalities in neural crest development in humans may result, *e.g.*, in craniofacial malformations. Teratogenicity of excessive vitamin A doses in different species including man has been also well established.
Thus, in principle, the new findings might suggest a potential of glyphosate or glyphosate-based herbicides to cause teratogenic effects by a mechanism similar to that one of retinoic acid / vitamin A also in humans. In fact, it seems to be the intention of the authors to establish just this link and to support the presumption of teratogenicity, most likely against the background of extensive use of glyphosate-containing herbicides in genetically modified soybean and other crops in South America. However, the conclusions drawn in this paper are not supported by Germany because of inherent weaknesses of the models used, methodical deficiencies and in particular because of the lacking evidence of teratogenicity based on both animal studies and epidemiological data.

Suitability of the models
Mechanisms of development are highly conserved in the evolution among vertebrates and *Xaenopus laevis* offers a number of advantages to study them as well as to recognize disturbances of normal developmental stages by xenobiotics. The chicken embryo has been also used for such purposes, although less frequently. However, even though these test systems are valuable tools in basic research in embryology and developmental physiology, they are certainly not standard models in routine toxicological testing for human safety. Similar developmental steps and mechanisms can be assumed throughout the vertebrate classes but there are big differences in toxicokinetics of chemicals between organisms with external development of the embryo (in water, in eggs) and those with internal growth and development in the uterus. Testing for developmental toxicity and teratogenicity in mammalian species that are much more similar to humans than frogs or chicken is more reliable and, therefore, is of higher relevance for human risk assessment. For pesticides, such higher tier studies are always required. In developmental toxicology, the described amphibian or avian test systems might be useful for screening new substances with unknown properties or to elucidate the mode or mechanism of action behind effects that were observed in standard studies in mammals but are not suitable to replace such studies.

Methodical deficiencies avoiding extrapolation to real world exposure of humans
Exposure of pregnant women to glyphosate (herbicides) would be mainly by the dermal or inhaltive route. Dietaty exposure would be of minor importance because residues are generally low and oral absorption will not exceed 30 %. In contast, treatment of frog or chicken embryos by cultivation in a medium containing the herbicide or, even more rigorously, by direct injection into the embryo are highly artificial routes of exposure. These experimental conditions do not reflect human exposure, kinetics (absorption, distribution, excretion and

their time course) in the mammalian organism or factors such as the placenta bareer in the pregnant female. In no case, exposure of the developing human fetus *in utero* would be similar to that one of the frog or chicken embryos in the study by Paganelli et al.
Quantitative comparison of the exposures is hardly feasible. It is not sufficient to state that tested concentrations for treatment of embryos or in further published *in vitro* experiments were below the intended or prescribed field spray concentrations. Comparison to internal doses received by exposed humans would be of greater interest but, still, could not provide sufficient information on *in utero* exposure.

Teratogenicity in mammals
As explained above, assessment of the teratogenic potential of a pesticide is mainly based on developmental toxicity in mammals, usually in rats and rabbits. As for all toxicological endpoints, the respective database for glyphosate, as compared to other active substances, is extremely huge. In the 1998 DAR on glyphosate that was prepared to support evaluation for first Annex I listing, a total of 7 studies in rats and 5 studies in rabbits are reported and a few more have been performed since then. No evidence of teratogenicity was obtained and in particular no craniofacial malformations were observed that one would expect if neural crest alterations by xenobiotics occurred in an *in vivo* experiment in mammals. For effects of this type, a clear dose-response can be assumed because the extent of malformations would depend on the severity of neural crest damage. In the developmental toxicity studies in rats and rabbits, exaggerated doses were employed and the absence of craniofacial effects even at these high dose levels can be taken as proof that the findings that were observed under very artificial conditions in *Xaenopus laevis* and chicken embryos are of no relevance for human risk assessment. The numerous available reproduction studies with glyphosate with a different mode of exposure (dietary vs. gavage administration) also failed to reveal a teratogenic potential.

The study authors suggest a possible bias because most part of the regulatory studies were provided by industry. This argument cannot be accepted since it is not likely that developmental effects would have not been reported for glyphosate but for other substances. (Classification and labelling for teratogenicity is mostly based on industry-sponsored studies.) In case of glyphosate, there is a number of companies producing and marketing this compound which nearly all provided an own (more or less complete) toxicological database. Thus, the absence of teratogenicity was confirmed in studies from different laboratories.
Furthermore, reproduction and developmental toxicity of a Roundup formulation (containing 36 % of glyphosate) was investigated in independent and published studies in Brazil (Dallegrave et al., 2003; Dallegrave et al., 2007). In these studies, effects on fetuses or pups were

more pronounced than with glyphosate alone and occurred at lower dose levels but, again, there were no craniofacial malformations.

Observations in humans

In spite of long-lasting use of glyphosate-based herbicides worldwide, no evidence of teratogenicity in humans has been obtained so far.

Indeed, there have been a few reports in local newspapers (and widely distributed via the internet) on a possible increase in malformations in newborn children in certain regions of Argentina and other countries in South America where pesticides (and among others glyphosate) are extensively used in particular on genetically modified soybean and corn. To our knowledge, there is no scientific confirmation of these reports so far. The study authors refer to a recent paper by Benitez Leite et al. (2009) suggesting a possible impact of exposure to agrochemicals on the frequency of malformations (among others, craniofacial) in a rural area in Paraguay. However, the database was rather small and confined to children born in one hospital. A relation to pesticide exposure was suspected but glyphosate was not mentioned in this article.

Even if there were indications for an increase in malformations because of extensive exposure to pesticides in South America, the state authorities in these countries would be responsible to initiate more in-depth investigations. Taking into account the very different application conditions and the uncertainties with regard to the plant protection products and human exposure, such findings would not automatically give rise to concern about the safety of glyphosate-based herbicides in Europe.

References

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2. Impact on risk assessment with regard to possible environmental effects

Experimental set up and findings

The experiments described in Paganelli et al. (2010) are in-vitro investigations on the effects of the herbicidal active substance glyphosate and of a commercial formulation thereof on the

embryonic development of frogs (African clawed frog *Xenopus laevis*) and chickens (White Leghorn strain).
The employed commercial formulation was stated to be "Roundup Classic" with 480 g glyphosate salt/kg, a product not authorized and distributed in Germany under this trade name. Therefore, the exact product composition is not known to us, but presumably corresponds to the original Roundup® formulation with 480 g IPA salt of glyphosate (360 g Glyphosate acid/kg product) and a blend of co-formulants containing polyethoxylated alkylamines (POEA) in the range of 150 g POEA/kg formulation. The uncertainty arising from the assumptions on the actually employed substances should be kept in mind when evaluating the results of the investigations described in the paper by Paganelli et al. (2010).
In the reported in-vitro investigations, frog embryos in the two-cell stage where exposed either to the commercial formulation of Glyphosate or directly injected with glyphosate alone. In a similar set up, chicken eggs were injected with Glyphosate.
The results of the trials show a downregulation of specific neural markers and alterations in the differentiation of primary neurons in the treated embryos compared to the controls. Marked effects were observed on neural crest cells, resulting in disrupted development of neural, skeletal and cartilage structures. Comparable response patterns were observed for frog embryos exposed to the glyphosate formulation and for embryos injected with glyphosate, although not all effects could be elicited and some response were weaker when glyphosate was injected alone.

Relevance of the observed in-vitro toxicity of glyphosate/glyphosate formulated products for the outcome of risk assessment procedures

As stated above the findings resembled teratogenic effects that occur in vertebrates either in maternal deficiency of vitamin A or in excess of retinoic acid. Regarding the relevance of the results for non-target species possibly exposed to plant protection products (PPP) containing glyphosate, the pertinence of the chosen experimental set up and especially the resulting exposure scenario in the discussed paper are of crucial importance.
Since amphibian species often release their eggs in open water, frog embryos might be exposed to contaminated water even if shielded by a gelatinous layer. Therefore, a possible upscaling to field situations of the observations achieved in part of the in-vitro experiments by Paganelli et al. (2010) might be correct. This is in our opinion the case for the results of one of the experimental set ups investigating the effects of whole frog embryo exposure to diluted PPP containing glyphosate and co-formulants.
If correctly interpreted, an exposure of the frog embryos to a diluted "Roundup Classic" formulation in proportion 1/5000 elicits clear toxic responses. As stated by the authors, this corresponds to an exposure to 430 µM of Glyphosate/L test solution, or, recalculated for the mole mass of 169,1 g/M, to 72 mg Glyphosate/L test solution.

For the purpose of this comment, we will name the ecotoxicological endpoint "*Xenopus laevi*s embryo development" and the lowest tested concentration of 72 mg/L as "surrogate ECx" for Glyphosate in this test.

The highest authorized Glyphosate application rate in Germany is 3600 g Glyphosate/ha. The maximal concentration that can be found in surface waters due to drift deposition after PPP field application can be calculated accounting for standard drift rates. In Germany, no direct overspray of water bodies is allowed, but maximum water concentrations after overspray might be found in the table below corresponding to a the distance from the field margin of 0 meters.

Active substance:		**Glyphosate**				
Application rate		**3600g a.i./ha**				
Spraydrift szenario / percentile:		**agriculture/90**				
distance (m)	**Deposition via Drift**		**Predicted Environmental Concentration Surface Water** [Σ Sum D + V (µg/L)]			
	(%)	**(µg/L)**	**conventional technique**	**90% Red.**	**75% Red.**	**50% Red.**
0	100	1200,00	1200,00	-/-	-/-	-/-
1	2,77	33,24	33,24	3,32	8,31	16,62
5	0,57	6,84	6,84	0,68	1,71	3,42

The relevant toxicity endpoint in the current risk assessment for Glyphosate is the *Skeletonema costatum* EC50 = 640 µg Glyphosate/L, clearly higher than the reported effect concentrations of 72 mg Glyphosate/L for frog embryos. The accepted Toxicity to Exposure Ratio for impairment of algal growth is ≥10. Following TER-values can be calculated:

relevant toxicity:	***Skeletonema costatum* EC_{50} = 640 µg/L**			
relevant TER:	**10**			
	TER-values (calculated)			
Distance (m)	**conventional technique**	**90% Red.**	**75% Red.**	**50% Red.**
0	0,5	-/-	-/-	-/-
1	19	192	77	38
5	94	936	374	187
Management needed:	**no**			

Similar calculations can be performed employing the reported endpoint by Paganelli et al. (2010) and a conservative acceptable TER of 100:

relevant toxicity:	*Xenopus laevis* EC_x = 72 000 µg/L			
relevant TER:	100			
	TER-values (calculated)			
Distance (m)	**conventional technique**	**90% Red.**	**75% Red.**	**50% Red.**
0	60	-/-	-/-	-/-
1	2166	21660	8664	4332
5	10526	105263	42105	21052
Management needed:	**no**			

As can be seen in the calculated TER values, an acceptable high margin of safety can be assumed when comparing the actually authorized application rates in Germany to the toxicity values reported by Paganelli et al. (2010). These figures might change quickly if higher application rates are authorized in other states, especially in view of the massive use of Glyphosate in genetically modified crops and/or direct overspray scenarios.

Therefore, we appreciate the claims raised by the authors on the need for a comprehensive understanding of the effects of the world-wide massively employed PPP with glyphosate on human health and on non target organisms.

We currently do not support the national authorizations of glyphosate-based PPP with a specific class of co-formulants, the polyethoxylated alkylamines (POEA). As also shown in the here commented paper of Paganelli et al. (2010), similar effects were observed in embryos exposed to "Roundup Classic" or injected with glyphosate alone, although not all effects could be elicited and some response were weaker when glyphosate was injected into the embryos. This could be a result of the different exposure design or derive from a minor toxicity of the tested active substance compared to the more toxic product with co-formulants. The presence of co-formulants from the class of the polyethoxylated alkylamines has been identified to drive the acute toxicity of glyphosate formulations for several aquatic species.

In the process of national registrations, Germany currently requires additional tests on the potential long term toxicity of PPP formulated with Glyphosate and POEA for aquatic organisms (tests with invertebrates, fish screening assay for the detection of possible endocrine disrupting properties and an extended amphibian metamorphosis assay with Xenopus laevis). Given the massive utilisation of Glyphosate-based herbicides, we do hope that the results of the required tests will address some of the concerns raised on the safe use of these products.