SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENVIRONMENTAL CHEMISTRY
POPULATION DYNAMICS
AGRI-ECOLOGY
CONSERVATION

Received
9 November 2012

Accepted
11 January 2013

Published
24 January 2013

Correspondence and requests for materials should be addressed to C.A.B. (bruehl@uni-landau.de)

Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline?

Carsten A. Brühl[1], Thomas Schmidt[2], Silvia Pieper[3] & Annika Alscher[1,4]

[1]Institute for Environmental Sciences, University Koblenz-Landau, Campus Landau, Fortstraße 7, D-76829 Landau, Germany, [2]Harlan Laboratories Ltd., Zelgliweg 1, CH-4452 Itingen, Switzerland, [3]Federal Environment Agency, Wörlitzer Platz 1, D-06844 Dessau, Germany, [4]Innovative Environmental Services (IES) Ltd., Benkenstrasse 260, CH-4108 Witterswil, Switzerland.

Amphibians, a class of animals in global decline, are present in agricultural landscapes characterized by agrochemical inputs. Effects of pesticides on terrestrial life stages of amphibians such as juvenile and adult frogs, toads and newts are little understood and a specific risk assessment for pesticide exposure, mandatory for other vertebrate groups, is currently not conducted. We studied the effects of seven pesticide products on juvenile European common frogs *(Rana temporaria)* in an agricultural overspray scenario. Mortality ranged from 100% after one hour to 40% after seven days at the recommended label rate of currently registered products. The demonstrated toxicity is alarming and a large-scale negative effect of terrestrial pesticide exposure on amphibian populations seems likely. Terrestrial pesticide exposure might be underestimated as a driver of their decline calling for more attention in conservation efforts and the risk assessment procedures in place do not protect this vanishing animal group.

Amphibians represent the prime example of the modern biodiversity crisis since they are the most threatened and rapidly declining vertebrate group, disappearing from different habitats on a global scale[1,2]. Competition with alien species, increased ultraviolet radiation, global warming, emerging infectious diseases, habitat loss due to land use changes and pollution are discussed as potential stressors[3–6]. With the clearing and conversion of grasslands and forests, agricultural area has become one of the largest terrestrial biomes on Earth, occupying more than 40% of the land surface[7], representing an essential habitat for amphibians. However, croplands in particular receive high chemical inputs to control pests, weeds and fungal infections leading to pesticide exposure, which could be another driver for the observed amphibian decline[8,9]. In contrast to birds and mammals, for amphibians to date no specific risk assessment is required for the registration of a new pesticide product. However, negative effects of pesticides on amphibians are probable since their skin is highly permeable to allow gas, water, and electrolyte exchange with the environment. Indeed dermal uptake processes of chemicals have been shown to be two orders of magnitude faster than in mammals[10], suggesting that for terrestrial amphibian life stages present in crop fields pesticide uptake through the skin might represent a likely exposure route[11]. Furthermore, with a life cycle that encompasses aquatic and terrestrial phases as well as migrations to and from spawning waters, amphibians are exposed to toxicants in two environments[12,13]. Aquatic environments receive pesticide inputs by un-intended pollution events where minute amounts enter water bodies by spray drift or run-off. This results in known pesticide effects on larval stages including endocrine disruption by atrazine[14] and increased mortality from environmentally relevant glyphosate exposure[15]. Terrestrial habitats like crop fields or fruit orchards on the other hand receive intentional pesticide applications at full rates. Although pesticides exposure of terrestrial amphibian life stages migrating and foraging in agricultural fields is likely[5,16], data on effects are scarce and of limited use for risk evaluation[11] as requested from EU authorities in the renewal process of the legislation on pesticide regulation[17]. A recently published summary report on the toxicity of pesticides to amphibians commissioned by the European Food Safety Authority identifies considerable research needs especially in the area of terrestrial exposure and effects[13].

To fill this knowledge gap, we studied the effects of seven pesticides (four fungicides, two herbicides and one insecticide) on juvenile European common frogs *(Rana temporaria)* in an agricultural overspray scenario. Juvenile frogs were exposed to three application rates (the recommended maximum label rate, 0.1× and 10× the label rate, rate: amount of pesticide per area) on moist soil. Since amphibians come into contact with pesticide formulations instead of pure active ingredients, we tested ready formulated products containing additives. We included the pyraclostrobin formulation Headline, a fungicide with the main formulation additive solvent naphta (67%), demonstrated to be highly toxic for *Bufo cognatus* juveniles[18], as a positive control. We added a different, unregistered formulation of pyraclostrobin with a lower solvent naphta content (<25%) to evaluate the effect of formulation additives versus the active ingredient and selected five additional products from the common pesticides for cereals and orchards in Germany or Switzerland.

Results

Acute mortality ranged from 100% after one hour to 40% after seven days at the recommended label rate of currently registered pesticide products (Fig. 1).

Since mortality reached 100% at the label rate applications for the fungicides Headline and Captan Omya, the higher 10× label rate treatment was not assessed to reduce animal testing. Similar levels of mortality, ranging between 40–60%, were observed for the other tested commercially available products. Three products showed a mortality of 40% after seven days at the lowest rate tested (10% of the label rate). The recorded mortality after 7 days for juvenile European common frogs differed largely between the two pyraclostobin formulations. Whereas the commercially available Headline formulation caused 100% mortality just after 1 h at the label rate, the formulation with the lower naphta content (BAS 500 18F) revealed 20% mortality at the label and 10× label rate.

Discussion

Effects were not restricted to a specific class of pesticides and seem to be influenced not only by the active substance but also the formulation additives. Both pyraclostrobin formulations contained the same amount of active chemical but differed in the content of the main formulation additive of solvent naphta (67% versus <25%). Mortality dropped from 100% in the high naphta product to 20% in the formulation with the lower solvent naphta content, indicating that other chemicals in the formulation may play a major role in the effect size of pesticides. Our test species, the European common frog *Rana temporaria*, is more sensitive to Headline compared to *Bufo cognatus* an American toad species that revealed a mortality of 70% after 72 h[18] presumably due to skin properties that differ between toads and frogs.

Unlike in larval stages, where development is affected and deformities are observed[14,15], environmentally relevant pesticide exposure of terrestrial life stages resulted in direct mortality in our study. The observation of acute mortality in a vertebrate group caused by commercially available pesticides at recommended field rates is astonishing since 50 years after the publication of Rachel Carson's *Silent Spring*[19] one would have thought that the development of refined risk assessment procedures and our growing understanding of environmentally effective chemicals would make such effects virtually impossible. Differences in the formulation additives revealed a great influence on toxicity, indicating the need to expand the evaluation from active chemical ingredients to entire products. However, it will be difficult to understand the role of additives since in many formulated products the exact chemical composition is not declared and percentages of additive chemicals are only indicated as 'proprietary ingredients'.

We studied a 'realistic worst-case' exposure scenario for single pesticides with direct overspray on a natural soil substrate. Although interception by crops may reduce exposure, repeated pesticide applications in a growing season might result in multiple contacts with a variety of products. Also, modeling approaches suggest that factors influencing juvenile and adult survival are most significant for the persistence of amphibian populations, and a 40% annual mortality is expected to cause a substantial decline[20].

The most toxic compound in our study, the top-selling fungicide Headline, is currently applied on 90 different crops from wheat in Canada to soy-beans in Argentina and its use may increase further due to proclamations of increase in crop yield and attractive discounts[21]. At present, several thousand pesticide products are

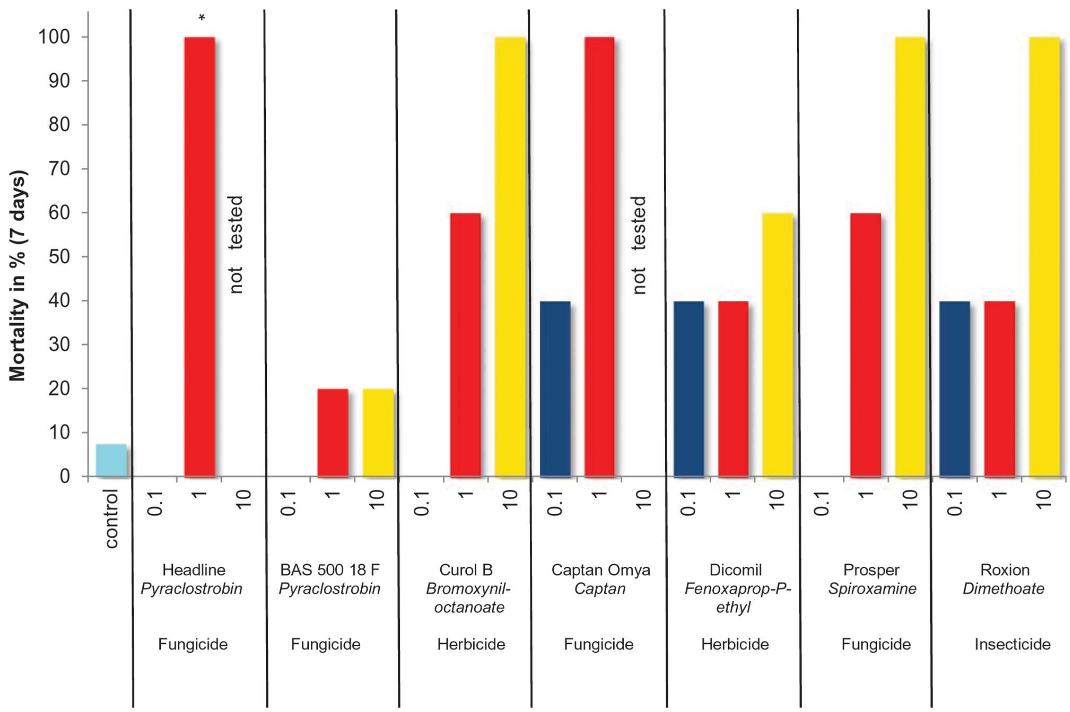

Figure 1 | Mortality of juvenile European common frogs *(Rana temporaria)* after seven days following an overspray exposure for seven pesticides at 0.1×, 1× and 10× the label rate (formulation name, active substance and class are given). Twice the 10× label rate treatment was not tested since the label rate already resulted in 100% mortality (see Methods section for more details). * 100% mortality occurred within 1 h. 0.1× label rate, label rate, 10× label rate.

registered globally and more than 2.3 million tons are applied on a major proportion of the land surface each year[22]. The demonstrated toxicity at recommended label rates of the few tested registered products resulting in mortality from 40% to 100% after seven days for juvenile European common frogs is alarming. Additionally, 40% mortality was observed for three registered products at 10% of the recommended label rate and therefore large-scale negative effects of terrestrial pesticide exposure on amphibian populations seem likely. Thirty-two of the 75 amphibian species occurring in Europe are associated with arable land according to the IUCN[13] and for some species movements in this landscape coincide with pesticide applications[13,16]. Especially amphibians that migrate to aquatic spawning habitats reveal high population declines[5,23] and pesticides might be a major threat for these species when crossing agricultural areas. Pesticide effects on terrestrial life stages of amphibians are so far not accounted for in amphibian conservation strategies where currently disease is discussed as a key factor[24]. We suggest that pesticides effects in cropped areas should be incorporated in landscape scale analyses for conservation management of amphibians.

Our results also indicate that existing risk assessment procedures for pesticide regulation are not protecting amphibians. Since amphibians are considered sentinel species for environmental and human health[25], our results might even have implications for other taxa or entire ecosystems. It is therefore imperative to understand the underlying mechanisms of the toxicity of pesticides for amphibians to obtain a realistic estimate of the extent of their impact and to reconcile agricultural practice and amphibian conservation efforts.

Methods

Ethics statement. The experiments described here were performed at Harlan Laboratories Ltd. (Itingen, Switzerland), an AAALAC-accredited laboratory in accordance with the Swiss Animal Protection Law. This research project was approved by the Veterinary Agency of the Swiss Cantonal Authorities (authorization for animal experiments no. 411 (August 4th 2011)). Animals were collected with approval of the Swiss Cantonal Department of Construction and Nature Protection.

Study conduct. We collected 150 juvenile *R. temporaria* on 5 August 2011 from the nature protection area "Tal" between CH-4467 Rothenfluh and CH-4469 Anwil. The juveniles were kept in Macrolon containers (Type III H (area 820 cm^2), UNO Roestvaststaal BV, Nederlands). At one side of the containers, an area of 30×3 cm was replaced by a mesh (mesh size: 1 mm) to increase ventilation within the containers. The containers were covered with a mesh lid (mesh size: 1.5 mm). The holding containers were filled with an approx. 3 cm layer of standard soil (Soil 2.3, LUFA Speyer, Germany) planted with barley seedlings (*Avena sativa*, sort: Eunovaa). Two pot shards (diameter: 5–7 cm) provided shelter and a petri-dish (diameter: 9 cm) filled with water was added. The containers were regularly watered with tap water from above. The juvenile frogs were fed with fruit flies *(Drosophila hydei)*, crickets *(Acheta domestica, Gryllus assimilis)* or white woodlouse *(Trichorhina trementosa)* obtained from commercial suppliers every two days. Frog density varied between eight to ten individuals in the holding phase.

For exposure and during the study conduct the animals were kept individually for a maximum of 7 days in Macrolon containers (Type II (area: 375 cm^2), UNO Roestvaststaal BV, Netherlands) covered with a mesh for ventilation. The test containers were filled with a 2 cm layer of standardized soil (Soil 2.3, LUFA Speyer, Germany), without vegetation that was kept moist from below to avoid leaching of the tested pesticide products from the soil surface. The frogs were fed every two days with fruit flies (*Drosophila hydei*) and crickets (*Acheta domestica*). The test containers were kept in a climate chamber (temperature 20 ± 2°C, relative humidity 75 ± 15%, 16 : 8 hours day:night).

The bottom part of the containers was removable to apply only the soil substrate and not the entire container. During application, the test organisms were confined to the substrate within a wire cage ($21 \times 15 \times 5$ cm, mesh size 4 mm). The applications were conducted with a laboratory track sprayer (Schachtner Fahrzeug- and Gerätetechnik, Germany) using commercial flat fan nozzles (TeeJet 8001 EVS, TeeJet Spraying Systems Co., USA). This sprayer produces similar spray deposits as a commercial application with a tractor using a boom sprayer for arable fields regarding droplet size and density. The sprayer was calibrated for each application in order to reach a spray volume of 200 L/ha on the soil substrate taking the interception of the wire cage into account. Directly after application, the sprayed container bottom parts with the confined juvenile frogs were inserted in the test containers and the wire cages were removed.

Three rates (0.1×, 1× and 10× label rate) of seven pesticides were tested. Since in the field amphibians come into contact with formulations instead of pure active ingredients, we tested ready formulated products (supplementary information, Table S1). The pyraclostrobin formulations Headline and BAS50018 F were provided by BASF SE (Crop Protection - Ecotoxicology, Limburgerhof, Germany). All other products were commercially available in Switzerland or Germany.

A maximum of five individuals were exposed per treatment. However, to minimize the number of test animals we used a step-wise approach: First, three juvenile frogs were exposed individually to the 0.1× field rate. If they did not show any treatment effects after 24 h, the remaining two individuals were also exposed. We used the same approach in all treatment rates for all pesticides. If the first three test organisms died, no further testing was conducted at this and at higher treatment rates. Accordingly, mortality at the higher treatment rates was assumed to also reach 100%. In parallel to each of the four consecutive spray application runs we used a control of 10 individuals treated with water only. Mortality was determined one, two and four hours after application and afterwards daily until the end of the study (day 7). Raw data are presented in Table S2 and Figure S3 (supplementary information).

1. Stuart, S. N. *et al.* Status and trends of amphibian declines and extinctions worldwide. *Science* **306**, 1783–1786 (2004).
2. Pounds, J. A. *et al.* Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* **439**, 161–167 (2006).
3. Collins, J. P. & Storfer, A. Global amphibian declines: sorting the hypotheses. *Divers. Distrib.* **9**, 89–98 (2003).
4. Blaustein, A. R., Wake, D. B. & Sousa, W. P. Amphibian declines - Judging stability, persistence and susceptibility of populations to local and global extinctions. *Conserv. Biol.* **8**, 60–71 (1994).
5. Becker, C. G., Fonseca, C. R., Haddad, C. F. B., Batista, R. F. & Prado, P. I. Habitat Split and the Global Decline of Amphibians. *Science* **318**, 1775–1777 (2007).
6. Sodhi, N. S. *et al.* Measuring the Meltdown: Drivers of Global Amphibian Extinction and Decline. *PLoS ONE* **3**, e1636, doi:10.1371/journal.pone.0001636 (2008).
7. Foley, J. A. *et al.* Global Consequences of Land Use. *Science* **309**, 570–574 (2005).
8. Mann, R., Hyne, R. V., Choung, C. & Wilson, S. Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environ. Pollut.* **157**, 2903–2927 (2009).
9. Hayes, T. B. *et al.* Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? *Environ. Health Persp.* **114**, 40–50 (2006).
10. Quaranta, A., Bellantuono, V., Cassano, G. & Lippe, C. Why Amphibians Are More Sensitive than Mammals to Xenobiotics. *Plos One* **4**, e7699. doi:10.1371/journal.pone.0007699 (2009).
11. Brühl, C. A., Pieper, S. & Weber, B. Amphibians at Risk? Susceptibility of Terrestrial Amphibian Life Stages to Pesticides. *Environ. Toxicol. Chem.* **30**, 2465–2472 (2011).
12. Todd, B. D., Bergeron, C. M., Hepner, M. J. & Hopkins, W. A. Aquatic and terrestrial stressors in amphibians: A test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. *Environ. Toxicol. Chem.* **30**, 2277–2284 (2011).
13. Fryday, S. & Thompson, H. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. EFSA Supporting Publications: EN-343 pp. 348 (2012). http://www.efsa.europa.eu/en/supporting/pub/343e.htm
14. Hayes, T. *et al.* Herbicides: Feminization of male frogs in the wild. *Nature* **419**, 895–896 (2002).
15. Relyea, R. A. The Lethal Impacts of Roundup and Predatory Stress on Six Species of North American Tadpoles. *Arch. Environ. Contam. Toxicol.* **48**, 351–357 (2005).
16. Berger, G., Pfeffer, H. & Kalettka, T. *Amphibienschutz in kleingewässerreichen Ackerbaugebieten* (Conservation of amphibians in agricultural lamndscapes rich in small water bodies). (Natur & Text, Rangsdorf, 2011).
17. Opinion of the Scientific Panel on Plant protection products and their Residues on a request from the Commission related to the revision of Annexes II and III to Council Directive 91/414/EEC concerning the placing of plant protection products on the market - Ecotoxicological studies. *EFSA Journal* **461**, 1–44 (2007). www.efsa.europa.eu/en/efsajournal/doc/461.pdf.
18. Belden, J., McMurry, S., Smith, L. & Reilley, P. Acute toxicity of fungicide formulations to amphibians at environmentally relevant concentrations. *Environ. Toxicol. Chem.* **29**, 2477–2480 (2010).
19. Carson, R. *Silent Spring*. 368 pp. (Houghton Mifflin, 1962).
20. Relyea, R. A. Amphibians Are Not Ready for Roundup®. 267–300. In Elliott, J. E., Bishop, C. A. & Morrissey, C. A. Wildlife ecotoxicology: forensic approaches. (Springer, New York, 2011).
21. Hooser, E., Belden, J., Smith, L. & McMurry, S. Acute toxicity of three strobilurin fungicide formulations and their active ingredients to tadpoles. *Ecotoxicology* **21**, 1458–1464 (2012).
22. Grube, A., Donaldson, D., Kiely, T. & Wu, L. Pesticide industry sales and usage: 2006 and 2007 market estimates. 1–41 (2011). Environmental Protection Agency, Washington. http://www.epa.gov/opp00001/pestsales/
23. Denoël, M. Newt decline in Western Europe: highlights from relative distribution changes within guilds. *Biodivers. Conserv.* **21**, 2887–2898 (2012).
24. Fisher, M. C. *et al.* Emerging fungal threats to animal, plant and ecosystem health. *Nature* **484**, 186–194 (2012).

25. Burkhardt, J. G. *et al*. Strategies for assessing the implications of malformed frogs for environmental health. *Environ. Health Persp.* **108**, 83–90 (2000).

Acknowledgements

We would like to thank Thomas Eltz, Susan. G. Letcher, Manuel E. Ortiz Santaliestra, Emma. J. Rosi-Marshall and Ralf Schulz for their comments on earlier versions of the manuscript. This study was co-funded by the Federal Environment Agency (UBA), Germany (FKZ 3709 65 421).

Author contributions

CAB planned and supervised the project and wrote the manuscript. TS organized the experiment and arranged all necessary permissions from relevant authorities. SP provided information on pesticides. CAB, TS and SP selected the pesticides. AA performed the experiment and data analysis. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Brühl, C.A., Schmidt, T., Pieper, S. & Alscher, A. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? *Sci. Rep.* **3**, 1135; DOI:10.1038/srep01135 (2013).