The ecotoxicological effects of a glyphosate-based herbicide on anuran larvae and their predators

Theses of the Ph.D. dissertation

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1. Introduction

Each year millions of tons of pesticides are used worldwide, and considerable amounts reach non-agricultural habitats due to wash-off by rain, wind and inappropriate use (Pereira et al. 2009), endangering ecosystems surrounding these areas (Giesy et al. 2000). Amphibians are considered the most threatened vertebrate group today (Wake & Vredenburg 2008), with more than 40% of the species being at risk (IUCN, 2016). The causes of destruction are the same as the processes underlying the global biodiversity crisis, namely habitat loss, climate change, increasing UV radiation, the appearance of alien predators, utilization as food, diseases and pollution (e.g. Hayes et al. 2010; Hof et al. 2011).

In nature xenobiotics are usually found at sublethal concentrations (e.g. Battaglin et al. 2009), however, these can also have serious consequences for organisms (Bridges 1999). Furthermore, pesticides can enhance the effects of other biotic and abiotic stress factors (Sih et al. 2004), therefore, pesticide contamination can have large effects on individuals, but also on ecological patterns and processes (Relyea 2005a).

Glyphosate-based herbicides are among the most frequently applied pesticides worldwide (Relyea 2005b; Grube et al. 2011), and glyphosate is one of the three most often detected anthropogenic chemicals in freshwater ecosystems (Pérez et al. 2011; Mörtl et al. 2013). These are broad-spectrum pesticides, which usually contain two main components, the glyphosate and some kind of surfactant (Giesy et al. 2000). One of the most commonly applied surfactants are the polyethoxylated tallow amines (POEA).

Previous studies showed that glyphosate-based herbicides are toxic to amphibians (Mann & Bidwell 1999). At sublethal concentrations, they can slow development, reduced size, and can also affect the behaviour and body shape of tadpoles (Howe et al. 2004; Relyea 2012; Moore et al. 2015).

2. Aims

In our experiments we investigated the effects of a glyphosate-based herbicide (Glyphogan® Classic, GC; Monsanto Europe S.A., Brussels, Belgium) on larvae of two anuran species, the agile frog (*Rana dalmatina*) and the common toad (*Bufo bufo*). Amphibians are especially suitable for examining effects of different chemicals on non-target organisms (Linder et al. 2010), however, amphibians have remained understudied in respect to the ecotoxicity of environmental contaminants for a long time (Johnson et al. 2017). The aim of my research was to answer the following questions:

1. Does sensitivity to GC change through development? Does exposure beyond the sensitive period enhances the effects of GC?

2. Does GC affect the two typical top predators of temporary water bodies, larvae of southern hawker (*Aeshna cyanea*) and adult smooth newts (*Lissotriton vulgaris*)?

3. Does GC affect tadpole behaviour? Do these responses resemble those given to the presence of a predator? Do semio chemicals from predators alter the effects of GC? Does GC inhibit the response to the presence of predators?

4. Is the surfactant responsible for most of the toxicity of GC? Does the simultaneous presence of semiochemicals from predators increase the harmful effect of the components?

5. In case of GC, how reliable and accurate are the estimated LC_{50} values of a single toxicity test? Do differences in the experimental setup affect the estimated toxicity of GC?

6. Does the applied experimental venue affect results of ecotoxicological tests? Do the effects of GC on survival, development, body mass, morphology and behaviour of tadpoles differ between experiments performed in the laboratory and in outdoor mesocosms?

3. Material and methods

We collected individuals used in the experiments as eggs from freshly laid egg-clutches, and transported them to the laboratory at the Júliannamajor Experimental Station of the Plant Protection Institute (Centre for Agricultural Research, Hungarian Academy of Sciences), Budapest. Until hatching, we kept clutches separately in containers filled with reconstituted soft water (RSW, APHA 1985). We started our experiments once tadpoles reached the free-swimming state (developmental stage 25 according to Gosner 1960).

We captured dragonfly larvae and adult male smooth newts using dip-nets and plastic funnel traps, and transported them to the Juliannamajor Experimental Station. We kept dragonfly larvae individually and newts in groups of 4 or 5 in containers filled with RSW, and fed them with bloodworms (*Chironomus* sp.) or *Tubifex tubifex* worms every other day until the start of experiments. For feeding predators and testing predatory activity we collected some more freshly laid egg clutches of the agile frog, and kept eggs separately in the laboratory in 10-L boxes. We reared hatching tadpoles at low temperature and high density to slow their growth, so that they remained small until predation tests.

In the laboratory, in the age-dependence experiment, we kept the common toad tadpoles individually in 1.2-L plastic boxes at 18 °C and a 12: 12 h light: dark cycle. We changed water every third day, while maintaining the initial GC concentrations (0, 2 or 4 mg a.e./L of glyphosate).

In the experiment comparing the toxicity of the components of GC, we reared the common toad and the agile frog tadpoles in 5-L plastic boxes at 19 °C and a 13.5: 10.5 h light: dark cycle, without water change. We exposed tadpoles to 0, 1, 2 and 4 mg a.e./L glyphosate, or 0, 0.44, 0.88 and 1.74 ml/L POEA, or to the combinations of the two components (1 mg a.e./l glyphosate + 0.44 ml/l POEA; 2 mg a.e./l glyphosate + 0.88 ml/l POEA; 4 mg a.e./l glyphosate + 1.74 ml/l POEA).

In the repeatability experiment, we kept the agile frog tadpoles individually in 2-L plastic boxes at 16 °C and a 12: 12 h light: dark cycle in the first experiment. We exposed tadpoles to 0, 0.009, 0.03, 0.24, 1, 2 or 6.5 mg a.e. glyphosate/L. Animals received semiochemicals from predators daily, and on the third day we replaced the water in the boxes. In case of the common toad, we kept 14 tadpoles in 11-L boxes at 18 ° C, 12: 12 hours light: dark cycle. We used 0, 1, 2, 3, 4, 5, 6, 7 or 8 mg of a.e./l glyphosate concentrations, we did not changed the water, but semiochemicals from predators were added daily to the rearing boxes. In the repeated experiment, in case of the agile frog, we held experimental conditions identical to those in the first experiment but only applied three herbicide concentrations (0, 2, and 6.5 mg a.e. glyphosate/L) in combination with the predator treatments. However, in case of common toad, tadpoles were placed individually in 1.2-L boxes, we exclude the predator treatment, and we changed the water after three days, maintaining the initial glyphosate concentrations (0, 2 or 4 mg of a.e./L).

When we investigated the effects of the experimental venues, we kept the agile frog tadpoles individually in 2-L plastic boxes at 16 °C and a 12: 12 h light: dark cycle at glyphosate concentrations of 0, 2, or 6.5 mg a.e./L. In this experiment we changed the water three times a week, and tadpoles were received semiochemicals from predators every day.

During the water change we used separate nets for each GC concentrations and predator treatments to avoid contamination with the herbicide and transmission of species-specific chemical cues. After the water change, we fed tadpoles with chopped and slightly boiled spinach *ad libitum*. For the predator treatments we used individually kept dragonfly larvae, fed with two naïve conspecific tadpoles every other day. We added water from the predator boxes to each experimental container assigned to a predator treatment, while in control treatments, we added the same amount of RSW.

To investigate the susceptibility of predators, we kept predators individually in 3-L plastic boxes, in a randomised block design. Boxes contained a small plastic pot and a wooden stick as hiding and perching sites. At the start of the experiment we filled boxes with 2 L RSW containing GC or 2 L clean RSW. We changed water two times a week, maintaining

the initial GC concentration. We fed predators with two naïve agile frog tadpoles and *Tubifex tubifex* worms every other day.

In the outdoor mesocosm experiments we used 90-L containers. Two weeks before the start of the experiments, we filled containers with 65 L of tap water and covered boxes with mosquito net lids to prevent colonization by macroinvertebrates. Two days later, when the chlorine left the water, we added 40 g of dried beech (*Fagus sylvatica*) leaves and 1 L of pond water to each container to enhance the growth of bacteria, phytoplankton and zooplankton. We placed automatic temperature loggers (HOBO) into 3 randomly chosen experimental containers to record water temperatures during the experiment. If the experiment included a predator treatment, we equipped tubs with a predator cage made of a plastic tube covered with mosquito nets on both ends. This prevented predators injuring focal tadpoles but allowed tadpoles to sense the presence of predators via chemical cues. One day before the start of experiments, we placed predators into cages in the predator treatments, whereas in mesocosms assigned to no-predator treatments the cages remained empty. We fed predators with two predator-naive tadpoles three times a week. To prevent feeder tadpoles to escape, we removed cages from the tubs during feeding. To equalize disturbance, we also lifted empty cages in no-predator treatments.

For the mesocosm experiment examining the susceptibility of predators, we used the same type of plastic tubs as in the experiment with tadpoles. We filled containers three weeks before the start of the experiment with 65 L tap water and covered them with mosquito net lids immediately after filling. One day later we added 1 L of pond water and 40 g of dried beech leaves to each container. We placed automatic temperature loggers into 3 randomly chosen experimental containers. To provide perching sites, we placed small plastic ladders into the mesocosms positioned vertically and reaching just below the water surface. We fed predators every other day with two small tadpoles and approximately 200 mg *Tubifex tubifex* worms. Because in this experiment tadpoles were in the tubs only for a short time, we added 3 large snails (*Lymnaea stagnalis*) to each mesocosm to prevent excessive periphyton growth.

In the statistical analyses, I used general linear models (GLM), generalized linear models (GZLM), multivariate general linear models (MGLM), linear mixed models (LMM), generalized linear mixed models (GZLMM), Bayesian generalized linear models, Spearman rank correlations, Kruskal–Wallis tests, Tukey's honest significant difference tests (Tukey's HSD), Firth logistic regressions.

4. Summary of results

1. We found that younger common toad tadpoles were more sensitive to GC, than the older ones. Furthermore, tadpoles exposed to GC during the majority of their larval period developed slower than tadpoles exposed only at the early stages of their development.

2. Exposure to GC had no effect on any life-history trait we measured either in larval southern hawkers or in adult male smooth newts, even at chronic exposure to the high concentration. We also found that neither chronic nor acute exposure to GC significantly affected predatory activity.

3. We found that GC can affect the behaviour of agile frog tadpoles, and some of these changes were similar to those induced by predators. We observed that at high GC concentration, tadpoles reduced their activity and hid more, while at the lower concentration they moved upward in the water column.

4. Our results confirmed that out of the two examined components, the POEA is the one that is primarily responsible for the harmful effects of GC on amphibians. In case of the survival of the tadpoles, we found that the presence of glyphosate slightly increased the lethality of the surfactant in both species.

5. We repeatedly determined the LC_{50} values for GC for agile frog and common toad tadpoles. In the case of common toad, the two experiments yielded nearly the same results despite differences in experimental design. However, in case of the agile frog, toxicity of GC differed between the two experiments despite almost identical experimental conditions.

6. Our results demonstrated that the type of experimental venue can have decisive effects on study outcomes. All measured life-history traits of tadpoles differed between the laboratory-based and the outdoor mesocosm experiment, and even more importantly, GC appeared to have opposite effects depending on the venue.

5. Discussion

We demonstrated that the susceptibility of amphibian larvae to GC is strongly age-dependent, so that the age of test animals has to be considered when planning ecotoxicological studies (Howe et al. 2004). Younger common toad tadpoles were more sensitive to GC than older ones in all measured life-history traits, while more developed tadpoles seemed to be more tolerant. Furthermore, tadpoles that were exposed to GC for a long time period developed

slower than tadpoles that were treated only at the beginning of their larval development. Nevertheless, we did not observe a similar effect to GC depending on the duration of exposure either in body mass or in survival. Our results suggest that if the application of pesticides would occur after the initial stages of tadpole development, that would be highly beneficial from the perspective of amphibian conservation, because tadpoles would be exposed to pesticides for a shorter time, and would not get into contact with the herbicide during their most sensitive early development.

In contrast, the predators of tadpoles seemed to be resistant to GC, since we did not find significant effects on their survival, body mass change, or predatory activity. These results are consistent with previous findings (e.g. Relyea & Edwards 2010) and suggest that the tested predators may fulfil their top-down regulatory role in ponds contaminated with GC. However, our experiments lasted for a relatively short time, so that further studies are needed to clarify potential long-term effects of exposure to GC.

GC altered the behaviour and body shape of tadpoles in a similar way as what can be observed in the presence of certain predators. This suggests that the physiological background behind these changes is the same, and that the observed responses are manifestations of the same general stress reaction (Middlemis Maher et al. 2013). However, because the physiological mechanism behind these alterations are unknown, further detailed investigations are required.

Of the two tested components of GC, POEA was primarily responsible for the toxic effects. In our experiment, we found significant effects only in treatment groups where the surfactant was present. The presence of glyphosate slightly increased the lethality of the surfactant in both species, but a similar effect on body mass of surviving tadpoles was not detectable. For this reason, during the authorization process of new pesticide formulations, it would be imperative to examine not only the active ingredients' harmful effects but to also scrutinize the toxicity of the excipients.

Our results demonstrated that the experimental setup and the experimental venue can considerably influence results of ecotoxicological studies. Our results show that the sensitivity of agile frog tadpoles to GC significantly differed between repeated laboratory experiments, while in case of common toad, the estimated LC_{50} values were nearly equivalent. This supports the hypothesis that larvae of different populations of amphibians may largely vary in their susceptibility to pesticides (Cothran et al. 2013). The type of experimental venue also had a large effect on the outcome: GC had an opposite effect on body mass, development time and morphology of tadpoles in the two venues, furthermore, GC was less lethal to tadpoles

reared in outdoor mesocosms than in the laboratory. Besides, the comparison of the results obtained from the two experimental venues suggests that results of standard laboratory-based tests may differ considerably from results of experiments carried out under natural or seminatural conditions. Unfortunately, due to the scarcity of similar studies, we need further investigations to tell how general our findings are, and to determine the environmental factors that have the strongest effects on the outcome of experiments.

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