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EFFECT DURATION AND SIDE-EFFECTS OF PERSISTENT PESTICIDES

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1. SCIENTIFIC BACKGROUND AND OBJECTIVES

Pesticides – used worldwide in crop protection for decades– are important representatives of possible environmental contaminants[1]. They may pollute natural habitats, causing increased concern on chemical environmental load[2] and on biological diversity.[3] A special attention is paid to the presence of water pollutants and their toxic effects, since this medium is habitat for many micro- and macroorganisms. Human population may become chronically exposed to contaminants *via* the drinking water. Large amounts of applied herbicides may disrupt aquatic communities in both direct and indirect ways and may decrease their biodiversity.[4] Persistence is also a major and current topic in plant protection. Prior to the 70's – for enhancing effect duration – more stable and persistent active ingredients were desired in pesticide development, but recently – in order to decrease the environmental load – the aim is to limit persistency. Considering environment protection viewpoints, rapidly degradable and specific chemicals are being developed instead of those previous, long-lasting substances. Persistence of each chemical depends on several factors; as it may vary between wide limits. In this thesis I summarize our studies and comparison of pesticides that are persistent under certain environmental conditions. Some of them (e.g. atrazine, trifluralin acetochlor) are secondary persistent[5] substances, while toxin proteins (e.g. Cry1Ab) produced by genetically modified (GM) plants – protected from degradation in plant cells – show a different form of persistency feature as well[6]. Plant parts of transgenic, Cry-toxin producing corn, containing variable amounts of the toxin, may reach aquatic habitats, where the toxin may present hazard to non-target organisms as well.

Special attention is being paid to toxicological and ecotoxicological side-effect assessment during pesticide authorization procedures. Our aim was to examine the effect duration of possibly water contaminating pesticides, mainly on non-target organisms. In certain cases, applied pesticides may remain in the environment after application,

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[1] Palumbi, S.R. (2001): Humans as the world's greatest evolutionary force. *Science* 293:1786–1790.
[2] Barceló, D., Hennion, M.-C. (Eds.) (1997): Trace determination of pesticides and their degradation products in water, techniques and instrumentation in analytical chemistry, vol. 19, *Elsevier Science* B.V., Amsterdam
[3] Vitousek, P.M., Mooney, H.A.L.J., Melillo, J.M (1997): Human domination of Earth's ecosystems. *Science* 277:494–499.
[4] Folmar, L.C., Sanders, J.O., Julin, A.M. (1979): Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. *Arch. Environ. Contam. Toxicol.* 8: 269–278.
[5]Eljarrat, E., Barcelo, D. (2003): Priority lists for persistent organic pollutants and emerging contaminants based on their relative toxic potency in environmental samples. *Trac-Trends in Analytical Chemistry* 22 (10): 655-665.
[6] Székács A. és Darvas B. (2012): Comparative aspects of Cry toxin usage in insect control. *In*: Ishaaya, I., Palli, S. R. , Horowitz, R. (eds.): Advanced technologies for managing insect pests, Berlin, *Springer-Verlag*, pp. 195-230.

therefore, different organisms or even the human population may come in contact with them. These substances cause not only rapid, acute toxicity, but during continuous, chronic exposure at sub-lethal concentrations they may facilitate chronic diseases or amplify other effects. During the work, my goal was to investigate acute and chronic effects of certain pesticides detected in natural environmental samples using analytical and toxicological methods. Besides, in examinations modeling natural environmental aquatic habitats, effect duration and side-effects of persistent pesticides were also explored. Through all these studies, we tried to draw attention to the importance of the comprehensive authorization processes of recent pesticides, beyond the compulsorily tests on the one hand, and consideration of (eco)toxicological evaluations on the other hand.

2. MATERIALS AND METHODS

As a part of a national monitoring project, analytical and toxicological evaluation of soil, ground- and surface water samples originated by Békés County were performed. In total during the investigation 423 soil and 202 water samples were processed. After sample preparation (ultrasound and solid phase extraction), concentrations of pesticide active ingredients were detected using gas chromatography coupled with mass spectrometry. For the detection of herbicide active ingredient glyphosate, an immunoassay was utilized using a validated ELISA kit. To the toxicological tests soil samples were subjected to solvent extraction, while water samples were used as obtained. Evaluations of toxic effects on different populations of *Daphnia magna* were performed using the immobilization test (ISO 6341:1996, OECD 202).[7] To explore teratogenic effects of herbicide active ingredient glyphosate, its formulation ROUNDUP CLASSIC and formulating agent polyethoxylated tallowamine (POEA) *Danio rerio* embryo toxicity tests were executed *via* the OECD 236 protocol.[8]

Effect duration and side-effects of Cry1-and Cry3-toxins were detected in aquaria equipped with water and mud samples originated from natural environments (Duna and Lake Velencei). For investigation, transgenic and near isogenic lines of GM corns (*MON 810* and *DAS 59122-7*), cultivated at our institute, were used. Dried plant parts were put into equipped aquaria, and in specific intervals water and leave samples were taken for analytical and toxicological tests. Toxin concentration was followed on the basis of an immunoassay method (ELISA) and the toxic effects using acute and chronic (feeding) tests

[7] ISO 6341:1996: Water quality- Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)- Acute toxicity test
[8] OECD 236 (2013): OECD guidelines for the testing of chemicals, (July), pp. 1-22.

on model organisms (*Daphnia magna* water flea and *Aedes aegypti* mosquito larvae). To determine Cry1Ab and Cry34Ab1 toxins, quantitative (Abraxis LLC[9]) and qualitative (EnviroLogix[10]) ELISA-kits were used.

To assess effect duration of Cry4-toxin, aquaria were set up with water and mud from Hévíz, Körös and Lake Velencei, and *Bti*-formulations (VECTOBAC WDG and VECTOBAC 12AS) were applied into them. *Ae. aegypti* larvae were used to detect Cry4-toxin effect duration. During the test, in specific intervals, water samples were taken for analytical experiments, moreover survived larvae were removed every second day after mortality checking, and new individuals were put into the system. After sample preparation, Cry4-toxin contamination of water samples were determined by ELISA method developed by own laboratory.

3. RESULTS

Of the soil samples analyzed, 18% of the samples contained detectable contamination by one or more target compounds. Of the water samples analyzed, the contamination rate was found 38%. Contaminants mainly appeared in sublethal concentrations. The most common contaminant appeared to be atrazine. Besides pesticide residues, contaminant microelements were also detected, such as B, Se, Cu and As. Of the 14 microelements monitored, 18% and 53% contamination frequencies above the legal threshold value were detected in soil and water samples, respectively. Not surprisingly, the most contaminated samples arrived from industrial sampling points (74% of samples were contaminated). As expected, no toxic effect was observed in the *D. magna* immobilization test in the case of the vast majority of the samples. In contrast, significant or salient aquatic toxicity was detected in all soil and water samples heavily contaminated with pesticide residues and/or toxic microelements, indicating that these contaminants do cause toxicity on *D. magna*. In some cases, individual toxic effects occurred to be altered by interactions among contaminants in sublethal concentrations (e.g. Cu and diazinone). For exploring the real effects, further experiments are needed. However, the knowledge of the exact ingredients and the mechanism of effect still cannot be guaranteed. Based on our experiments, it can be concluded that the knowledge of the effects of each pollutant detected separately in the previous monitoring project is not sufficient for the exploration of their combined effects.

[9] Abraxis Cry1Ab/Ac ELISA kit (http://www.abraxiskits.com/moreinfo/PN510001USER.pdf)
[10] EnviroLogix QualiPlate™ Kit for Cry34Ab1 (http://www.envirologix.com/library/ap054insert.pdf)

Herbicide active ingredient glyphosate was detected in 26% of observed samples. Toxicological values of glyphosate vary highly; therefore, the evaluation is difficult. As expected, our two different *D. magna* populations showed differences in sensitivity to glyphosate. Exploration the toxicological characteristics of pesticide formulating agents has received special attention lately. Herbicide formulation ROUNDUP CLASSIC, its active ingredient glyphosate and its formulating agent polyethoxylated tallowamine (POEA) were examined on *D. magna* and *D. rerio* model organisms in both mortality and teratogenicity tests. In accordance with literature data, POEA was found to be the most toxic substance (LC_{50} for *D. magna* 0,8-5,1; for *D. rerio* 4,5-5,4 mg l^{-1}), followed by the formulation (LC_{50} for *D. magna* 15,3-33,6; for *D. rerio* 78-147 mg l^{-1}), while the active ingredient occurred to be the least toxic (LC_{50} for *D. magna* 100-1080; for *D. rerio* >9750 mg l^{-1}). High values of standard deviation on *D. magna* toxicity values caused by varying sensitivity of two different populations. High concentrations of POEA and ROUNDUP CLASSIC (above 20 and 100 mg l^{-1}, respectively) caused immediate coagulation on *D. rerio* embryos. At sublethal dose (near EC_{50} values) of the formulating agent and the formulation, several developmental malformations were observed in the embryos: mainly head deformations, inhibition of heartbeat or blood circulation, lack of pigmentation and edemas (pericardium- or yolk sac edemas). Due to the increase in incriminating data and extensive usage of chemicals mentioned above, the re-evaluation of these chemicals' environmental risk assessments and consideration of potential sublethal and mixture effects have become an important task for the future. Besides, revision and toxicological evaluation of inert ingredients should be an inevitable step during pesticide authorization and releasing processes.

Examinations with Cry1- and Cry3-toxins were performed in aquaria equipped with water and mud samples originated from natural environment (Duna river and Lake Velencei). Because of the natural water medium, matrix effects on ELISA test were substantial. During the test no toxin was detected in the water samples, and no effects were sen on the model organisms, either. Initial concentration of Cry1Ab-toxin in *MON 810* corn leaves (4986 ng g^{-1}) dramatically decreased during the first 24 hours in both aquaria (with 80% and 88% in Lake Velencei and Duna, respectively), and then the decrease continued at a lower pace. Similarly, toxin content of *DAS 59122-7* leaves decreased below limit of detection in both aquaria. In acute tests none of the observed corn stubble had mortality effects on water flea and mosquito larvae. To chronic tests, dried green leaves with higher toxin concentration were used. In food assessment the mortality of adults was

significantly higher in the test group (fed with Cry1Ab-toxin containing plant particles) than in the control group (fed with their standard food, algae), but there was no significant difference between transgenic and isogenic corn lines. Similarly, in case of *DAS 59122-7* corn, the survival of the control group was the highest, but there was no observed significant difference among test groups. The effect of the toxins on reproduction was assessed only with this corn line. The highest number of offspring occurred in the control group, but the difference between the isogenic and transgenic corn lines did not differ statistically. Experimental circumstances of reproduction tests on D. magna must be improved in order to be able to easily distinguish between the effects of corn feeding and toxins. With these tests the effects of toxin-containing plant particles could not be explained. It seems that the D. magna as a test organism is not an ideal choice for this exploration. Our long term aim is to select aquatic invertebrates with appropriate food preferance to separate and detect the exact effects of the toxin only.

Examinations of Cry4-toxin were performed similarly to the mentioned above. Three aquaria were set up with water and mud from Hévíz, Körös and Lake Velencei, then two types of *Bti*-formulations (VECTOBAC WDG and VECTOBAC 12AS) were put into them to detect effect duration using analytical and toxicological methods. Because there was no suitable commercially available analytical method for Cry4 toxin, an enzyme-linked immunosorbent assay (ELISA) was developed for quantitative determination of Cry4 toxin. As in natural water samples, significant matrix effects occurred. Preconcentration of aqueous samples by lyophilization resulted in low but reproducible recoveries (~26 %), and the practical LODs for Bti preparations VECTOBAC WDG granulate and VECTOBAC 12AS suspension were found to be ~170 ng ml^{-1} and ~900 ng ml^{-1} , respectively. The application rate for the aquarium test was chosen to be 0,4 mg l^{-1} for granulate and 1 mg l^{-1} for suspension determined by acute mortality tests.

The half-life of toxin – in case of VECTOBAC WDG - in surface water from Velence, Hévíz and Körös was found to be 3,8 ± 0,3, 4,5 ± 0,6 and 5,5 ± 1,5 days, respectively. The data showed that the initial 0,4 mg l^{-1} concentration of granulated formulation decreased by 5% and 13% by the 2^{nd} day in surface water obtained from Körös and Hévíz, respectively, continued to 18% and 44% by the 4^{th} day, and subsequently dropped below LOD. Mortality data of the mosquito larvae indicated further decrease in the toxin concentration. A similar trend was seen in surface water obtained near Lake Velencei. Concentration data determined by ELISA can be further extrapolated below the LOD on the basis of the larval toxicity test. It had to be considered, however, that larval mortality is not necessarily

directly proportional with the toxin concentration in the aqueous phase, as the larvae may uptake toxin from the sediment surface during feeding. According to results of the mosquito larval mortality test, mortality in the aquarium containing water and sediment from the Körös, of lesser organic matter content, remained nearly 100% up to the 7th day after the administration of the *Bti* preparation. The number of surviving larvae later commenced to increase and reached 52% by the last (11th) day of the experiment. In contrast, mortality in the aquarium containing water and sediment from Hévíz, rich in organic matter and forest litter, decreased to 83% by the 4th day upon application, followed by a further drop to 46% by the 9th day. The final mortality rate of 50% may have been affected by the fact that a thick layer of bacteria was formed on the water surface impeding the larvae to obtain fresh air. Similarly in case of Lake Velencei the mortality decreased continuously; the 55% by the 9th day dropped to 48% by the end of the test. Similar measurements using the suspension VECTOBAC 12AS formulation at an initial concentration of 1 mg l^{-1} were also carried out. As this concentration is only slightly above the practical LOD of the ELISA for VECTOBAC 12AS, the decrease of the formulation was not followed by ELISA, only by larval mortality. Moreover, the effect duration of VECTOBAC 12AS was considerably shorter (35% by the 4th day and ceased by the 7th day) in surface water from Velence, than that of VECTOBAC WDG. In summary, based on the mortality data it can be concluded that the efficacy of a given preparation is significantly affected by the organic matter content in the water phase. In waters rich in organic matter, larvae are likely to consume less toxin due to abundant nutrient availability than in waters poor in nutrients, and therefore, the efficacy of the preparation is also lower. The data also indicate that careful planning of the biological mosquito control methods, quantitative and qualitative survey of larval breeding sites and assessment of its characteristics are necessary because effective dosages and the required numbers of treatments at given locations can only be calculated by considering these factors as well.

4. NEW SCIENTIFIC RESULTS

1. The occurrence and toxic effects of pesticide residues and contaminating microelements that originated from samples of cultivated land were examined, as part of a complex soil monitoring project. It was concluded, for contaminants observed on *Daphnia magna*, that the toxicities of single contaminants differ from those of complex environmental samples. According to our experiments, the recent Hungarian Soil Monitoring System is insufficient for evaluation of the effects of complex environmental samples. Therefore, in order to detect the effect of the different combinations of contaminants in soil and water samples, a combination of analytical and biological methods is necessary.

2. Effects of a glyphosate based herbicide (ROUNDUP CLASSIC), its active ingredient glyphosate IPA-salt and its formulating agent (POEA) on different populations (laboratory and wild type) of *D. magna* were firstly examined. LC_{50} values were determined and variant sensitivity of different population was showed. According to the mortality tests, the toxicity values were as follows: glyphosate (360 and 900 mg/l) << ROUNDUP CLASSIC (18,7 and 20,6 mg/l) < POEA (1,28 and 3,3 mg/l).

3. Teratogenic effects of POEA formulating agent were firstly determined on *Danio rerio* embryos. Similarly to results on *D. magna*, the toxicity values were as follows: glyphosate (> 9750 mg/l) << ROUNDUP CLASSIC (90 mg/l) < POEA (5 mg/l). Besides LC_{50} values, in sublethal concentrations mainly head deformations, inhibition of heartbeat and blood circulation were detected.

4. I concluded that the toxin content of transgenic *Bt*-corn stubble (*MON 810* and *DAS 59122-7*) in aquaria containing freshwater from environmental habitats was decreased dramatically (more than 80%) within the first 24 hours, and it continued to diminish at a lower rate afterwards. Cry1- and Cry3-toxins could not be detected in water.

5 No acute effects of transgenic *Bt*-corn stubble (*MON 810* and *DAS 59122-7*) were exerted on *D. magna* and *Ae. aegypti*, although in chronic tests the mortality on adult water flea fed with corn was significantly higher than in the control group. Significant differences between transgenic and isogenic corn lines were not

observed. The highest number of offspring was detected in the control group; nevertheless there was no difference between transgenic and isogenic corn lines. I concluded that this experimental design needs to be improved in order to distinguish the effect of the toxin from the effects of food types.

6. For the first time in the case of Hungarian surface waters, an enzyme-linked immunosorbent assay (ELISA) was developed for the detection of the effect of duration and degradation of Cry4-toxin. The practical LODs for *Bti* preparations VECTOBAC WDG granulate (~170 ng ml^{-1}) and VECTOBAC 12AS suspension (~900 ng ml^{-1}) were determined. The DT_{50} of toxin – in case of VECTOBAC WDG – in surface water from Velence, Hévíz and Körös was found respectively to be: 3,8 ± 0,3; 4,5 ± 0,6; 5,5 ± 1,5 days. The results of mosquito-larvae test vary in different water samples, showing a high dependence upon organic matter content. It can be concluded that this method is suitable for detecting Cry4-toxin, but the sensitivity in the case of environmental samples needs to be developed further.

5. LIST OF SCIENTIFIC PUBLICATIONS

5.1. Scientific paper related to the subject of the dissertation

5.1.1. Proceedings in Hungarian

Fejes Á., Fekete G, Székács A. és Darvas B. (2010): Cry-toxin tartalmú kukoricapollen (MON 810 és DAS-59122) és néhány vízi szervezet (*Aedes aegypti, Daphnia magna*) kölcsönhatása. p. 61. *In.* Abs. 56. *Növényvédelmi Tudományos Napok, III. Géntechnológia- Növény-és Környezetvédelem Szimpózium*, Budapest

Székács A., **Fejes Á**., Mörtl M., Bokán K., Bánáti H., Fekete G. és Darvas B. (2011): A *glyphosate* környezet-egészségügyi hatásai. pp. 24-25. *In*: Darvas B. (szerk.) Abs. *I. Magyar Ökotoxikológiai Konferencia*, Budapest

Mörtl M., **Fejes Á**., Bokán K., Darvas B. és Székács A. (2011): A hazai növényvédő szer eredetű vízszennyezők és előfordulásaik környezeti mintákban. p.19 *In*: Darvas B. (szerk.) Abs. *I. Magyar Ökotoxikológiai Konferencia*, Budapest

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5.1.2. Proceedings in foreign language

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5.1.3. Scientific articles in Hungarian

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Darvas B., Bokán K., **Fejes Á.**, Maloschik E., Székács A. (2009): Növényvédő szerek környezetanalitikai és ökotoxikológiai kockázatai. *In.*: Németh A. (szerk.): Természetvédelem és ökológiai gazdálkodás. Budapest, *Magyar Biokultúra Szövetség* pp. 11-17.

Takács E., **Fejes Á.**, Fekete G., Darvas B., Székács A. (2010): Cry4 toxin hatóanyag vízi hatástartam- és lebomlásvizsgálata *immunoassay* és *Aedes aegypti* lárvateszt segítségével, *Acta Biol. Debr. Oecol. Hung*. 21:223.-232.

Darvas B., **Fejes Á.**, Mörtl M., Bokán K., Bánáti H., Fekete G. és Székács A. (2011): A *glyphosate* alkalmazásának környezet-egészségügyi problémái. *Növényvédelem*, 47: 387-401.

Fekete G., **Fejes Á.**, Székács A., Mörtl M., Zöldi V., Reisinger M., Darvas B. (2011): Csípőszúnyogok elleni védekezés Magyarországon. *Növényvédelem* 47(5):195-203. 10

5.1.4. Scientific articles in foreign language

Á. Fejes, E. Takács, G. Fekete, B. Darvas, B. S. Ferguson, D. Saxena and A. Székács (2012): Aquatic effect duration and degradation study of Cry4 toxin with immunoassay and *Aedes aegypti* larval biotest. *Aquatic Insects*, 34 (Suppl. 1): 211–226. IF: 0,496

A. Székács, M. Mörtl, G. Fekete, **Á. Fejes**, B. Darvas, M. Dombos, O. Szécsy and A. Anton (2014): Monitoring and biological evaluation of surface water and soil micropollutants in Hungary. *Carpathian Journal of Earth and Environmental Sciences*, 9 (3): 47-60 IF: 1,171*

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5.2. Other publications

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* In case of article published in 2014: mean of last five years impact factors

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Takács E., **Fejes Á.**, Klátyik Sz., Szántai-Kis Cs., Darvas B. and Székács A. (2014): Comparative toxicity assessment of a herbicide active ingredient with its formulating adjuvants on human cell lines. Abs. p.23. *In*: *Korean – Hungarian Workshop on Joint Research for Global Food Security: Ensuring Environmental and Food Safety*, Budapest

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