FINAL REPORT
Project ID: **K109865**
Mechanism-related teratogenic, hormone modulant and other toxicological effects of veterinary and agricultural surfactants

Principal Investigator: **Prof. András Székács**

The main purpose of the project has been a systematic evaluation of the toxicological effects of certain formulating agents used in veterinary drugs (VDs) and plant protection products (PPPs or pesticides) to assess the assumed biological inertness of these additives (adjuvants, surfactants). Toxicity evaluation has been carried out directly by testing of the active ingredients and the formulants (when available) or indirectly by comparing the biological effects of the active ingredients and their formulated products. Toxicity tests targeted on cytotoxicity, hormonal and genotoxic effects tested in *in vitro* assays on cell cultures or in *in vivo* biotests on indicator organisms. Assays were extended by chemical analysis of the target compounds – an approach that in given cases required analytical method development. Thus, the work comprised of the below approaches:

- *Overview of the surfactants used in VDs and in PPPs,*
- *Analytical method development and chemical analyses (for active ingredients, surfactants or biological indicators)*
- *Toxicity investigations of VDs and PPPs*
- *Aspects of risk assessment/authorization and practical applications*

Our research strategy followed the original research plan, participating researchers were those specified in the project plan, as well as several PhD students and young researchers. Instrument acquisition has been completed as planned. Cell culture work has been completed with the originally planned collaborating working groups at the Institute of Experimental Medicine of the Hungarian Academy of Sciences and the National Institute of Oncology, however, after the reorganization of the former groups, cellular tests were continued in-house and at the Nanobiosensoric Group at the Institute of Technical Physics and Materials Science, Centre for Energy Research of the Hungarian Academy of Sciences, as reflected also in the joint publications with these research groups. During the completion of the project 3 PhD and 8 BSc or MSc dissertations have been or are being completed.

As for the scientometric indices of the publication record of the project, 22 scientific articles and 2 book chapters were published (3 articles and 1 book chapter are to come out in print soon), with a cumulative impact factor of 41.062. In addition, 24 lectures and 22 posters have been presented at national and international conferences. Research results were published in leading periodicals of analytical chemistry, as well as environmental, agricultural and biological sciences e.g., *Int. J. Envir. Anal. Chem.*, *Sci. Total Environ.*, *Food Control*, *J. Food Comp. Anal.* and others (often D1 or Q1 ranked). Project results are summarized below in thematic order with the corresponding publications (scientific articles or conference presentations) listed after each section.

1. Overview of the surfactants used in VDs and in PPPs

Formulations applied in chemical plant protection, in animal husbandry and in veterinary practice contain various additives (co-formulants) besides the active ingredient(s). Among additives, classified into several groups by their function, adjuvants are a minor group, used for the primary purpose to enhance the biological effect. Thus, adjuvants (e.g., surfactants,

solvents, dispersing agents, activators, wetting or antifoaming agents, anti-evaporants, drift retardants, softeners, safeners, stabilizers, and penetrants) directly affect the efficiency of the formulations. A characteristic feature in the chemical structure of different surfactants is the simultaneous presence of hydrophobic and hydrophilic moieties. Surfactants are generally classified according to the type of their hydrophilic part; therefore, anionic, cationic, non-ionic, and amphoteric surfactants can be distinguished. The annual world production of surfactants was at 15 million tons in 2005 and rose substantially within a decade. Besides industrial and domestic application of various surfactants, the use in VDs and PPPs represents a substantial sector, as well. Surfactants in VDs (*Table 1*) are used for solubilization of the active ingredient through micellar dispersion, to enhance drug solubility and membrane permeability, prolong gastrointestinal residence time, and protect the active ingredient from luminal degradation and metabolism in the gut wall. Detergents as feed additives (*Table 2*) promote better digestibility and availability of nutrients. Surfactants in PPPs (*Table 3*) enhance the efficacy of formulations by increasing water solubility, bioavailability and biological activity of the active ingredients.

Table 1. Various types of surfactants used in VDs or disinfectants

Chemical name	Product name	Type	Producer/supplier	*CAS* number
Dioctyl sodium sulfosuccinate	Vedco Veterinary Surfactant	Anionic	Respa Pharmaceuticals Inc	577-11-7
Didecyl dimethyl ammonium bromide	Bromosept 50	Cationic	ABIC Biological Laboratories Teva Ltd	2390-68-3
Alkyl dimethyl benzyl ammonium chloride (C_{12-18}) (ADBAC)	Dec-quat 100	Cationic	Veltek Associates Inc	68391-01-5
Alkyl dimethyl ethyl benzyl ammonium chloride (C_{12-14}) (ADBAC)				85409-23-0
Polyethylene glycol (PEG) glyceryl stearate	Gelucire 50/13 Gelucire 50/02	Non-ionic	Gattefossé SAS	9011-21-6
PEG glyceryl laurate	Gelucire 44/14	Non-ionic	Gattefossé SAS	57107-95-6
PEG-8 caprylic/capric glycerides	Labrasol	Non-ionic	Gattefossé SAS	61791-29-5
12-Hydroxystearic acid-polyethylene glycol copolymer	Solutol HS 15	Non-ionic	BASF	70142-34-6
Sorbitane ester ethoxylate	Polysorbate 80	Non-ionic	Croda Americas, Inc.	9005-65-6

Table 2. Various types of surfactants used in feed additives

Chemical name	Product name	Type	Producer/supplier	*CAS* number
Sodium lignosulfonate	Arbo S01P Borresperse Na	Anionic	KemTek Industries Inc Borregard Ligno Tech	8061-51-6
Calcium lignosulfonate	Borresperse Ca	Anionic	Borregard Ligno Tech	8061 52 7
Linear calcium dodecylbenzene sulfonate	Rhodacal 60/BE	Anionic	Solvay & Rhodia	26264-06-2
Glycerol-polyethylene glycol ricinoleate	Volamel Extra Alkamuls SC/242	Non-ionic	Nukamel Solvay & Rhodia	61791-12-6
Alcohols, C_{8-10}, ethoxylated propoxylated	Antarox BL 225	Non-ionic	Solvay & Rhodia	68603-25-8

Table 3. Various types of surfactants used in PPPs

Chemical name	Product name	Type	Producer/supplier	*CAS* number
Alkyl (C_{8-10})-polyoxyethylene ether phosphate	Rolfen Bio	Anionic	Lamberti SpA	68130-47-2
POE alkyl phosphate ester				50769-39-6
Dioctyl sulfosuccinate sodium salt	Imbirol OT/NA/70	Anionic	Lamberti SpA	577-11-7
Sodium-alkyl polyglucoside citrate	Eucarol AGE-EC	Anionic	Lamberti SpA	151911-51-2
Sodium-alkyl polyglucoside sulfosuccinate (in aqueous solution)	Eucarol AGE 91/S K	Anionic	Lamberti SpA	151911-53-5
Sodium dodecyl benzene sulfonate	Agrosurf WP85	Anionic	Lankem Ltd	25155-30-0
Secondary alcohol ethoxylate	Tergitol 15-S-9	Non-ionic	Dow Chemicals	68131-40-8
POE (15) tallow amine formulated	Emulson AG GPE3/SSM	Non-ionic	Lamberti SpA	61791-26-2
Non-ylphenol polyethylene glycol ether	Triton N-57	Non-ionic	Dow	127087-87-0

Adjuvants (e.g., surfactants) and other co-formulants used in veterinary medicine, feed additives, as well as in pesticide formulations have long been classified as inert ingredients in the aspects of their required main biological effects of the pharmaceutical or pesticide product. Co-formulants, e.g., surfactants cannot exert such main effects *per definitionem*, as otherwise they would be also considered as active ingredients. Due to this definition, formulating

surfactants have been considered as ‘inert’ ingredients. In fact, this definition has been the cause, why formulants are considered – erroneously – entirely inert. Such “inertness” applies only to the main therapeutic/technological effect; it cannot warrant against unintended detrimental side-effects. The significance of potential effects of unidentified and assumedly inert pesticide ingredients on human and environmental health triggered our current research. The more complex the interaction of those substances with the potentially exposed organisms is, the broader the possibility of the occurrence of such side-effects becomes.

Klátyik *et al.* (2017) Authorization and toxicity of veterinary drugs and plant protection products: residues of the active ingredients in food and feed and toxicity problems related to adjuvants. *Front. Vet. Sci.*, **4**: 146.

Székács, A. (2017) Mechanism-related teratogenic, hormone modulant and other toxicological effects of veterinary and agricultural surfactants. *Insights Vet. Sci.*, **1**: 024-031.

2. Analytical method development and chemical analyses

2.1. Determination of active ingredients – residue analysis

As certain ecotoxicity tests of ours were carried out in water from Danube River, initial pesticide contamination levels were determined, major contaminants were identified. In addition to our gas chromatography method coupled with mass spectrometry (GC-MS) used in our previous monitoring studies, we also developed a liquid chromatography – mass spectrometry (LC-MS) method for measuring new polar active ingredients (e.g., neonicotinoids). Water samples were prepared by solid phase extraction (SPE) with graphitized carbon or Oasis HLB phases. Consequences of the withdrawals of earlier active ingredients were reflected in the contamination profiles observed. Thus, previously frequent contaminants e.g., acetochlor did not appear in the Danube in 2015. However, the previously banned (marked by * throughout the text) herbicide active ingredient atrazine*, persistent under anaerobic conditions in soil, was present in 91% of the surface water samples, although at low levels (0.6-1.2 ng/l), while its degradation product, desethyl-atrazine 4.4-4.8 ng/l was measured in 64% of the samples during May-July, 2017. Terbuthylazine and its metabolite desethyl terbuthylazine were also common pollutants at 3.8-41.1 and 6.2-10.2 ng/l, respectively, and carbendazim* appeared frequently (82%) at levels of 0.3-28.2 ng/l. Among neonicotinoid insecticides, thiamethoxam (TMX) and clothianidin (CLO) occurred at levels of 3.5-16.8 ng/l, mainly in the summer (June), in spite of the current ban against these compounds in seed coating. The chloroacetamide herbicide metolachlor* was detected at very high concentrations up to 1000 ng/l in the late spring – early summer period, and the organophosphate insecticide chlorpyrifos also showed a similar pattern of occurrence. Less frequently, dimethenamide and sulfotep* have also been found, and deltamethrin (widely used in mosquito control) metabolites were detected in the summer (July), and herbicide pendimethalin occurred as a rare contaminant. As seen, major pesticides in Danube River are herbicide residues, particularly those used in maize production, although insecticide residues may also occur in some periods at high concentrations. The results of our monitoring were published partly in summary articles and partly reported along with ecotoxicology studies (e.g., dissipation of glyphosate by algae, Klátyik *et al.* 2017). For the analysis of the Danube water samples spiked with the herbicide active ingredient glyphosate and its decomposition, we modified and validated a previously reported detection method. For this method, glyphosate and its main metabolite (aminomethylphosphonic acid, AMPA) was chemically modified (FMOC-Cl), subjected to solid phase extraction (SPE) with Oasis HLB phase and extract pre-concentration, and was detected with a chromatographic (HPLC-UV) method. The limit of detection (LOD) of glyphosate was 5 μg/l and recoveries ranged 83.5±6.0%.

Székács *et al.* (2014) Monitoring and biological evaluation of surface water and soil micropollutants in Hungary. *Carpathian J. Earth Environ. Sci.*, **9** (3): 47-60.
Székács *et al.* (2015) Monitoring pesticide residues in surface and ground water in Hungary – surveys in 1990-2015. *J. Chem.*, **2015**: Article ID 717948.

2.2. Determination of surfactants

As the effects of the active ingredients and the corresponding formulated preparations were found substantially different in many cases in ecotoxicity tests, we have developed analytical methods for the identification and quantification of the formulation agents. The formulating surfactant of the insecticide preparation APACHE (active ingredient: CLO) is a complex mixture of sulfonates, we characterized by our LC-MS method (*Fig. 1*). We determined its major components, and due to the absence of reference materials, we quantified their content by approximation: External calibration using a commercially available analogue compound (n-hexadecanesulfonic acid sodium salt) slightly underestimated the concentration values relative to data specified in the material safety data sheet of the formulated insecticide.

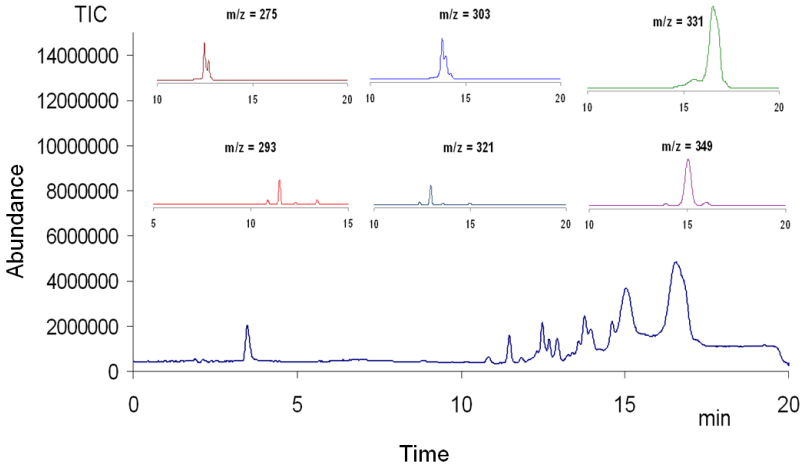

Figure 1. LC-MS chromatogram (total ion count, TIC) recorded by applying high buffer concentration (100 mM) and extracted ion chromatograms (*inserts*). The total ion chromatogram indicates a complex composition of alkanesulfonic acid derivative surfactants, with individual components of molecule ions (m/z) of 275, 293, 303, 321, 331 and 349 shown in the inserts.

Formulating surfactants in MOSPILAN (active ingredient: acetamiprid, ACE) and their decomposition in surface water samples (Danube) were also detected and monitored by the HPLC-UV method, and degradation of the surfactants were determined in the presence or absence of the active ingredient. The composition of linear alkylbenzene sulfonates (LASs) in MOSPILAN is almost the same as in commercially available detergent dodecylbenzene sulfonate mixtures, containing four major components characteristically separated chromatographically (retention time (Rt): 3.2, 3.5, 3.9 and 4.4 min, respectively). We compared the degradation of LASs in neat solution and in MOSPILAN both in distilled water and surface water from Danube, and determined the corresponding half-lives (DT_{50}s). As expected, LAS degradation is faster in Danube water, than in distilled water, but degradation in formulated MOSPILAN is slower than in neat solution of LASs. Thus, DT_{50} values of the four LAS components were estimated 10 and 2 days in distilled water in neat solution and in Danube water samples, respectively. Decomposition in distilled water occurred with a shallow slope and a residual level after 20 days, while in the Danube, it occurred rapidly and exhaustively. Decomposition of MOSPILAN

occurred with similar kinetics showing an initial lag phase, but with a somewhat steeper slope in Danube water samples, with estimated DT_{50} values of 28 and 20 days in distilled water and Danube water, respectively (*Fig. 2*). In line with literature data, the relative amounts of longer chain analogues were found to decrease over time. We also investigated the degradation of LASs in surface water (Danube) alone and in the presence of various neonicotinoids. TMX and CLO did not significantly affect the half-life of LASs, ACE exerted only a slight effect, while imidacloprid (IMI) and thiacloprid (TCL) significantly delayed degradation. Our results have been presented at international and national conferences, and following the *EMEC18 – 18th European Meeting on Environmental Chemistry* at Porto, Portugal, are intended to be published in a leading periodical *Science of the Total Environment*.

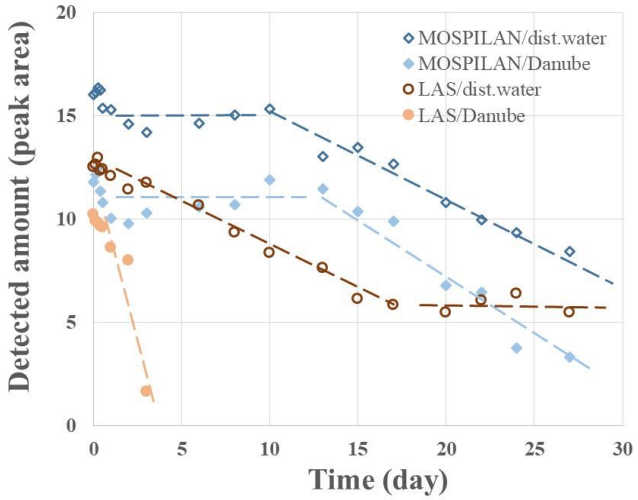

Figure 2. Decomposition of neonicotinoid type formulated insecticide MOSPILAN and its surfactant linear alkylbenzene sulfonates (LASs) in distilled water and surface water from the Danube. MOSPILAN in distilled water (◇) and in water from Danube (◆), LASs in distilled water (○) and in water from Danube (●).

The diversity and the wide polarity range of the chemical components represent a major difficulty in environmental analytical studies. GC methods are suitable for the measurement of narrow groups of surfactants, often only after chemical derivatization. Thus, we have tested several derivation processes for use in surfactant analysis. Among the additives, Nonit (dioctyl sulfosuccinate sodium salt) and Silvet Star (Trisil, polyalkylene oxide-modified heptamethyltrissiloxane) adhesion enhancers, as well as Triton X, Genamin and Sapogenate surfactants were analyzed. In the case of Nonit, the reaction with potassium iodide trifluoroacetic anhydride was tested, while in the other cases, our previously used silyl carbamate silylation process was applied for GC-MS measurements. The shelf life of the sulfonate derivative obtained is rather short (approximately 1 day), therefore, analysis has to be carried out immediately upon derivatization, or the method requires further development. A particular advantage is, however, that the detection of the derivative is feasible by electron capture detection, capable to provide very good LODs. For Silvet Star and the other surfactants tested, the silylated derivatives formed rapidly under mild conditions. Each oligomer was identified, and their distribution and ratios were determined on the basis of the mass spectra of their silyl ethers. The stability of the silyl derivatives could be assured with sufficient reagent excess, and could be further enhanced by the formation of tert-butyl dimethylsilyl esters. The results have been presented at international and national conferences, and the corresponding publication can be submitted after full validation of the procedures.

Takács *et al.* (2017) Effects of neonicotinoid insecticide formulations and their components on *Daphnia magna* – the role of active ingredients and co-formulants. *Int. J. Envir. Anal. Chem.*, **97** (9): 885-900.
Mörtl *et al.* (2017) Determination of surfactants used in agrochemicals. *Proc. 23rd International Symposium on Analytical and Environmental Problems* (Szeged, Hungary, October 9-10, 2017) pp. 62-66.
Mörtl *et al.* (2017) Determination of surfactants used in agrochemicals. *18th European Meeting on Environmental Chemistry (EMEC)* (Porto, Portugal, November 26-29, 2017)

2.3. Determination of retinoids in fish tissues

Retinoic acid (RA), as an active metabolite of vitamin A, is vital for vertebrate embryonic development, namely in somitogenesis, neurogenesis and organogenesis. Retinoic acid/retinol (RA/ROH) and 13-cis-RA/ROH ratios are useful parameters in teratogenicity studies to assess xenobiotic effects, thus, analytical determination of three retinoids by high performance liquid chromatography (HPLC) method was applied in our genotoxicity studies in zebrafish (*Daphnia magna*) (see 3.2.1, below). For analytical determination of retinoids in full body homogenates, fish were collected at different developmental stages and sizes of 2-4 hr eggs, 10-day juveniles (DR1), 10-14 mm (DR3) and 25-30 mm fish (DR6). The HPLC method was optimized with standard solutions of 13-cis-RA, RA and ROH. External calibration was used in the range between 15 and 20 ng/ml. For sample preparation, two extraction methods, using hexane – ethyl acetate (1:1) or ethyl acetate containing 0.5% tert-butylphenol, were assessed. ROH concentrations were found to be 0.035±0.003 and 0.289±0.039 μg/ml in groups DR3 and DR6, respectively, while RA and 13-cis-RA were found to be below LODs. Therefore, RA/ROH and 13-cis-RA/ROH ratios could not be calculated, and assessment had to rely on ROH levels detected. Nonetheless, this preliminary survey indicated that ROH (and possibly RA and 13-cis-RA, if detected by more sensitive analytical methods) are suitable biomarkers of xenobiotics (e.g. VDs or PPPs) in exposed zebrafish (*Danio rerio*).

Gyurcsó *et al.* (2017) Effects of glyphosate-based herbicides and their components on the embrional development of zebrafish (*Danio rerio*): Assessment of the role of retinoids. *Proc. 23rd International Symposium on Analytical and Environmental Problems* (Szeged, Hungary, October 9-10, 2017) pp. 420-425.

3. Toxicity investigations of VDs and PPPs

3.1. Veterinary drugs

3.1.1. Biotests, in vivo *assays*

Chemical substances used in various fields of agriculture (e.g., veterinary medicine or crop protection) represent relevant environmental loads, and their residues, metabolites and decomposition products possibly occur in the wastewater and can easily reach surface water. Residues of both of their active ingredients and formulating agents can occur as water contaminants or organic pollutants that need to be decomposed in wastewater. Dissipation of these substances needs to be followed in these water matrices, and technologies for their chemical or biological decomposition need to be developed.

To compare the toxicity of various active ingredients and formulations used in veterinary medicine, acute toxicity tests were performed on three test organisms (*Vibrio fischeri, Pseudokircheriella subcapitata, D. magna*). Results showed significant differences in the individual acute toxicity of various active ingredients and formulations used in veterinary medicine on *D. magna*. Sulfamethoxazole (SMX) and trimethoprim (TRI) were the least toxic investigated active ingredients; the evaluated EC_{50} values were 98.1±58.7 and 93.1±33.2 mg/l, respectively. The most toxic active ingredient was sulphaguadinin* (SGD) (EC_{50}=1.79±0.34

mg/l). Significant differences were observed in case of the toxicity of the investigated VDs containing SMX and TRI. SUMETROLIM was more toxic on *D. magna* (EC_{50}=106.2±54.9 mg/l) compared to COTRIUM-E. The combined toxicity was the highest when SMX and TRI were investigated together at SUMETROLIM equivalent concentrations compared to the formulated VDs.

In wastewater management the application of advanced oxidation processes is in the focus of interest due to their high efficiency in the removal of persistent organic pollutants. Ecotoxicological evaluation of treated SMX solutions was also carried out using above three test organisms. Additionally, the effects of the presence of H_2O_2 on the toxicity of 0.1 mmol/l SMX solutions oxidized during gamma irradiation (1 kGy, 2.5 kGy) was also assessed. The untreated SMX solution resulted in 5±1% inhibition on *V. fischeri,* while 30±2% inhibition was higher in both irradiated solutions and in the presence of H_2O_2. H_2O_2 showed significantly high inhibition on the investigated test organisms, with reduced inhibition at decreasing H_2O_2 concentrations on *V. fischeri* and *P. subcapitata* experiments. Evaluated EC_{50}s for *V. fischeri, P. subcapitata* and *D. magna* were 0.349, 0.251 and 0.064 mmol/l.

Experiments in the Institute for Energy Security and Environmental Safety, Centre for Energy Research, Hungarian Academy of Sciences verified that ionizing radiation can degrade antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) individually or in mixtures in aqueous solutions. Decomposition of these active ingredients alone, in mixture and in their formulated preparation SUMETROLIM was monitored directly and by bioassay using *Staphylococcus aureus* and *Escherichia coli* test strains. The results indicate that the technology is capable of degrading these antibiotics even when present in commercial pharmaceutical formulations such as SUMETROLIM. SMX was more susceptible to ionizing radiation as compared to TMP. The antibacterial activity of SMX and TMP was completely eliminated at 0.2 kGy and 0.8 kGy, respectively (*Fig. 3* A). However, when SMX and TMP were in a mixture the dose required to eliminate the antibacterial activity was 10 kGy implying a synergistic antibacterial activity when these are present in mixtures and an in the commercial formulation (*Fig. 3* B, C). These results imply that the synergistic antimicrobial activity of antimicrobial compounds with each other and with surfactants in pharmaceutical waste streams is a strong possibility. Therefore, antimicrobial activity assays should be included when evaluating the use of ionizing radiation for the remediation of pharmaceutical or municipal waste streams.

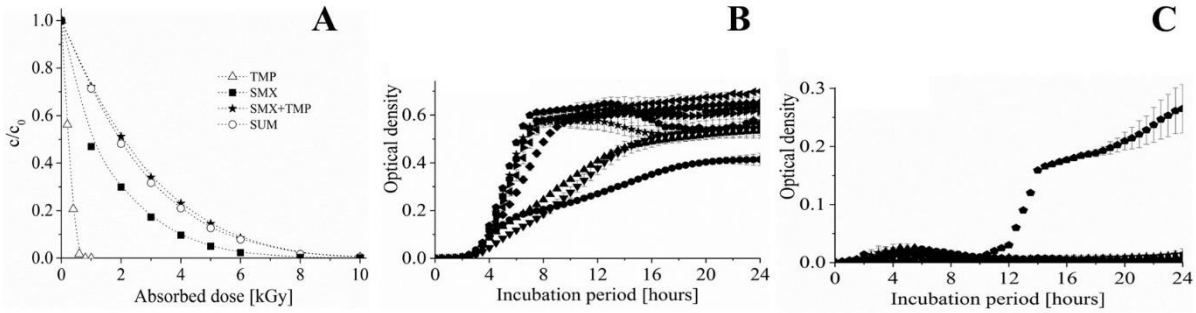

Figure 3. Degradation of sulfonamide antibiotics as VD active ingredients sulfamethoxazole (SMX) and trimethoprim (TMP), and preparation Sumetrolim™ (SUM). Degradation of SMX, TMP, SUM and SMX+TMP (5:1) mixture (**A**). The initial concentrations of SMX and TMP were 500 and 100 µmol/l, respectively. C = final concentration, C_0 = initial concentration. Multiplication of *Staphylococcus aureus* (**B**) and *Escherichia coli* (**C**) in the presence of SMX+TMP mixture previously exposed to varying ionizing radiation doses (● 0, ▲ 1, ▼ 2, ♦ 3, ◄ 4, ► 5, ⬢ 6, ★ 8 and ⬟ 10 kGy). The initial concentration of SMX+TMP was 500 µmol/l SMX + 100 µmol/l TMP.

In summary, our aquatic toxicity studies carried out with a VD SUMETROLIM on the great water flea (*D. magna*) indicated that the toxicity of the preparation was 25% higher than it would be expected from the individual toxicities of the active ingredients SMX and TRI, indicating a synergistic effect between the active ingredients or between formulating additives and the active ingredients. Decomposition of the active ingredients by ionizing radiation of chemical (H_2O_2) treatment could be followed by monitoring the ecotoxic effect, if toxicity of the decontamination agent was eliminated.

Klátyik *et al.* (2017) Comparative evaluation of veterinary active ingredients and formulations *ISAEP 2017*, poster

Székács, A. (2017) Mechanism-related teratogenic, hormone modulant and other toxicological effects of veterinary and agricultural surfactants. *Insights Vet. Sci.*, **1**: 024-031.

Sági *et al.* (2018) The impact of H_2O_2 and the role of mineralization in biodegradation or ecotoxicity assessment of advanced oxidation processes, *Radiat. Phys. Chem.*, **144**: 361-366.

Sági *et al.* (2018) Radiolysis of sulfonamide antibiotics in aqueous solution: Degradation efficiency and assessment of antibacterial activity, toxicity and biodegradability of products. *Sci. Total Environ.*, **622-623**: 1009-1015.

Sági *et al.* (2018) Elimination of antibacterial activity of sulfamethoxazole and trimethoprim by ionizing radiation. *J. Environ. Sci. Health A*, accepted for publication

3.1.2. Related findings with significance in veterinary biochemistry

The intestinal epithelium is an essential barrier against invading bacteria, oxidative stress and various chemical agents. Malfunction of the epithelial defense can lead to alimentary tract problems of concern both in human health and animal husbandry. A barrier integrity test methods based on transepithelial electrical resistance (TEER) was developed and utilized in the project for testing of xenobiotics including formulating surfactants at the University of Veterinary Medicine Budapest. TEER measurements on monolayers of an IPEC-J2 porcine intestinal epithelial cell culture were performed as an *in vitro* model to elucidate the mode of action behind modulatory effects of agricultural adjuvants. The effect of oxidative stress on barrier integrity and localization of transmembrane serine proteinase 2 (TMPRSS2) were studied on membrane inserts to estimate how the enterocyte-formed epithelial layer was affected by H_2O_2-induced oxidative stress. Peroxide-triggered enhanced paracellular permeability of the IPEC-J2 cell layer was accompanied by predominantly cytoplasmic occurrence of TMPRSS2 embedded in cell membrane under physiological conditions. Localization of TMPRSS2 in IPEC-J2 cells by immunofluorescent staining with Alexa 564 red fluorescent dye was proven useful in indicating oxidative stress (*Fig. 4*). Immunofluorescence staining experiments were analyzed using an Olympus IX73 inverted microscope with 100WHg fluorescent accessory obtained in the project. The results supported that reactive oxygen species can influence paracellular gate opening *via* multifaceted mode of action without involvement of β-catenin redistribution in adherens junction. The effects of 3-amidinophenylalanine-derived combined matriptase-1/matriptase-2 inhibitors on hepcidin production were also investigated in hepatocyte mono- and hepatocyte – Kupffer cell co-cultures, and hepcidin overproduction along with other biochemical markers was observed in hepatocytes upon treatment, supported by a molecular modeling study. Moreover, the function of Type II TMPRSS2, matriptase was evidenced to be inhibited by low molecular weight dipeptide amides and is expected also to be affected by other zwitterionic amino acid derivatives, as well as by membrane disrupting surfactants (e.g., tallow amine derivatives). For this purpose, the effects of formulated glyphosate-based herbicide components were also tested (see 3.2.1, below).

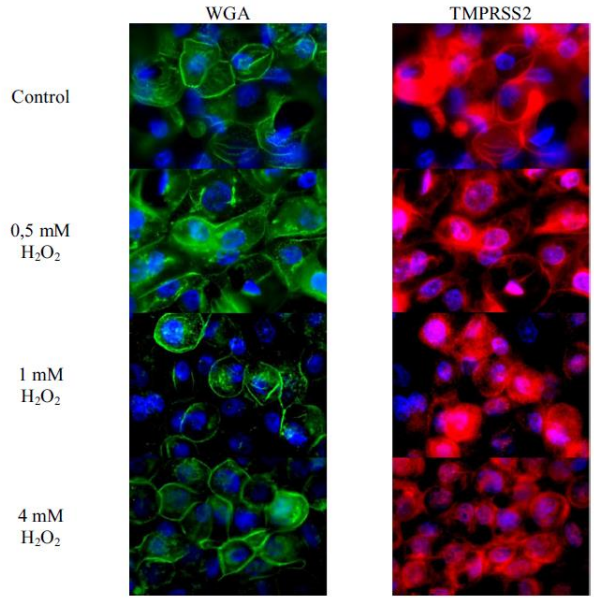

Figure 4. Subcellular immunolocalization of TMPRSS2 in non-tumorogenic IPEC-J2 cells cultured on polyester membrane inserts. Cell nuclei are stained blue (DAPI), membranes are labeled green with wheat germ agglutinin (WGA, Alexa 488, green), TMPRSS2 is labeled by red fluorescent dye (Alexa 564, red). Treatment with 0.5, 1 and 4 mM H_2O_2 for 1 h prior to staining changed TMPRSS2 localization and resulted in elevated cytoplasmic TMPRSS2 occurrence. Thus, Alexa 564 red fluorescent staining is proven to be an applicable indicator of oxidative stress through visualization of TMPRSS2 trafficking to cytoplasm induced by it.

Paszti-Gere *et al.* (2015) Changes in the distribution of Type II transmembrane serine protease, TMPRSS2 and in paracellular permeability in IPECJ2 cells exposed to oxidative stress. *Inflammation*, **38** (2):775-783.

Pászti-Gere *et al.* (2015) Reinforced epithelial barrier integrity via matriptase induction with sphingosine-1-phosphate did not result in disturbances in physiological redox status. *Oxid. Med. Cell. Longev.*, **2016**: Article ID 9674272, 7 pages

Pászti-Gere *et al.* (2018) 3-Amidinophenylalanine derived matriptase inhibitor can up-regulate hepcidin production *in vitro*. *Oxid. Med. Cell. Longev.*, submitted

3.2. Pesticide formulations

3.2.1. Biotests, in vivo assays

Pesticide formulations trigger load on environment due to their components, thus toxicological evaluation of active ingredient and co-formulants is essential for an appropriate environmental risk assessment of pesticides. Individual and combined toxic effects of various neonicotinoid- and glyphosate-based pesticides were investigated on aquatic organisms.

On the basis of the OECD 202 acute immobilization tests on *D. magna*, significant differences were observed in the toxicity of neonicotinoid active ingredients (CLO, TCL, TMX) and their formulations (ACTARA SC, APACHE 50 WG, CALYPSO 480 SC) tested. Among the examined active ingredients, TCL was the most toxic (EC_{50} = 5–13.5 mg/l), followed by TMX (EC_{50} = 93–159 mg/l) and CLO (EC_{50} > 340 mg/l). CLO at the concentration of 236 mg/l resulted in 25% mortality, while at its water solubility limit, 340 mg/l caused immobilization of 38.3% of the *D. magna* juveniles. The average 48-hr EC_{50} values determined for the investigated formulations were 2.2–3.2-fold higher than the reported values on the basis of the MSDSs. In contrast to the toxicity of the active ingredients, investigated formulation APACHE

50 WG (containing 50% CLO) proved to be the most toxic ($EC_{50[AI]}$ = 11.4±3.7 mg/l), CALYPSO 480 SC (containing 48% TCL) was less toxic ($EC_{50[AI]}$ = 27.0±9.5 mg/l), while the toxicity of ACTARA 240 SC (containing 24% TMX) was the lowest ($EC_{50[AI]}$ 226.7±68.2 mg/l) for the applied aquatic test organism. The toxicity of active ingredients TCL and TMX were 2.7 and 1.8 times higher than their formulations investigated, respectively, while APACHE 50 WG was found to be 46.5 times more toxic that explained by its active ingredient, CLO. Probably the applied formulating agents are responsible for the enhanced adverse effect for APACHE 50 WG, and possibly also for the reduction in the toxicity of CALYPSO 480 SC and ACTARA SC, relative to their active ingredients.

Individual and combined toxic effects of glyphosate-based herbicides (ROUNDUP CLASSIC, MEDALLON PREMIUM) and their components (glyphosate, POEA and alkyl polyglucoside surfactants) were also determined on *D. magna* test organism based on the OECD 202 guideline. Assays were performed on our laboratory culture and individuals provided in Daphtoxkit F test. In each experiment our own laboratory culture showed an increased sensitivity to the tested substances. Surfactant POEA exerted the highest toxicity on immobilization with 48 hrs EC_{50} = 2.7–5.1 mg/l. Alkyl-polyglucoside, surfactant in MEDALLON PREMIUM, was less toxic (48 hrs EC_{50} = 50.5–88.6 mg/l). The EC_{50} value for 48 hrs for glyphosate was 80.8–409.1 mg/l. For ROUNDUP CLASSIC, for both *D. magna* culture the adjusted toxicity (48hrs $EC_{50\,[AI]}$ = 8.88 - 24.8 mg/l) was higher than the toxicity of the active substance. For the MEDALLON PREMIUM, toxicity of glyphosate was higher in the case of our laboratory culture (48h $_{EC50\,[AI]}$ = 166–199.3 mg/l), in contrast in the case of the culture of the kit the order of the toxicity showed similarity for the ROUNDUP CLASSIC.

Chronic effects of glyphosate and ROUNDUP CLASSIC on *D. magna* reproduction were also determined by OECD 211 guideline. Chronic effects on reproduction were determined at 5 concentrations with a concentration range of 18.75–300 mg/l and 0.625–10 mg/l for glyphosate and ROUNDUP CLASSIC, respectively. For each concentration, 10-10 individuals were individually examined. Reproduction was observed for all individuals in the control group and in the lowest concentration group of glyphosate. At the highest concentrations only the half of the introduced *D. magna* reproduced. In the control group and in the three lowest concentrations of the formulated product, 70% of the individuals examined gave birth to offspring, whereas at the highest tested concentration, reproduction was observed in the case of only 2 individuals. The average daily number of offspring was 3.6±1.4 in the control group, while in the glyphosate treated groups, from the lowest concentration to the highest level, 3.5±2.0; 5.1±2.0; 2.4±2.0; 3.2±2.0 and 3.3±1.4. The average daily number of offspring was 3.0±1.4 in the control group and 3.6±1.4 for the highest concentration in the ROUNDUP CLASSIC treated groups; 5.4±0.8; 3.9±2.6 and 3.0±0.0.

Retinoids regulate differentiation, development and embryogenesis of vertebrates, and induce changes in their endogenous levels eventually lead to teratogenic effects. Vitamin A or all-trans-retinol (atROH) is converted enzymatically in a sequential process to all-trans-retinoid acid (atRA), and is further isomerized into its isoform 13-cis-RA. The progression of this enzymatic process triggers numerous cellular effects, the retinoic acid signaling pathway (RA pathway), and is an indicator of its activation. The RA pathway has been implicated in various developmental processes, e.g. during early embryonic development, retinoids act as important morphogens, and participate in regulating apoptosis, differentiation and cell fate specification. Surfactants used in glyphosate-based herbicides and the active ingredient glyphosate itself have been indicated to interfere with the formation of retinoids and the RA pathway. Thus, we have determined levels of atROH, atRA and 13-cis-RA in *D. rerio* embryos and young fish emerging.

Lethal and sublethal teratogenic effects of the herbicide active ingredient glyphosate, four formulated glyphosate-based herbicides (MEDALLON PREMIUM, ROUNDUP CLASSIC,

TOTAL, GLYPHOS) and four surfactants (POEA, sodium-alkyl polyglucoside citrate and sulfosuccinate, POE alkyl phosphate ester) frequently applied in herbicides were investigated based on the OECD 236 guideline on *D. rerio* embryos. Herbicide active ingredient glyphosate was found the least toxic on embryos. Its LC_{50} was higher than 9.8 g/l (glyphosate concentration in 2% ROUNDUP CLASSIC applied in agricultural practices). Formulated herbicides were 44-336 times more toxic than glyphosate, that were considered to be due to the chemical characteristics and quantity of the surfactants, since the nominal concentration of the active ingredient did not differ in these products. The least toxic one among formulated herbicides was MEDALLON PREMIUM (LC_{50}: 220.6±5.6 mg/l) that contains an alkyl polyglucoside surfactant; while the most toxic were ROUNDUP CLASSIC (LC_{50}: 90.0±4.5 mg/l) and GLYFOS (LC_{50}: 28.8±3.2 mg/l) that contain a non-ionic surfactant POEA. The pure surfactants were identified as the most toxic components: their LC_{50} values were 232–2438 times lower than that to glyphosate. Below or near the LC_{50} values, deformities, edema (pericardial), inhibition of heartbeat and circulation were the most frequently detected non-lethal malformations in every treatment.

Takács *et al.* (2017) Effects of neonicotinoid insecticide formulations and their components on *Daphnia magna* – the role of active ingredients and co-formulants. *Int. J. Envir. Anal. Chem.*, **97** (9): 885-900.
Gyurcsó *et al.* (2017) Effects of glyphosate-based herbicides and their components on the embryonal development of zebrafish (*Danio rerio*): Assessment of the role of retinoids. *ISAEP 2017*. poster.

Individual and combined toxic effects of these two glyphosate-based herbicides and their components were also determined on three floating single-celled green algae species (*Desmodesmus subspicatus, P. subcapitata*, *Scenedesmus obtusiusculus*). Effect of glyphosate was investigated on an anaphoric blue-alga species (*Anabaena flosaquae*), as well. Reproduction inhibition assays were performed based on the OECD 201 guideline, inhibition was determined by optical density (OD) measurement and for *D. subspicatus* by determination of chlorophyll-a content. Toxicity of test substances in descending order was the POEA> ROUNDUP CLASSIC > glyphosate. The 72h EC_{50} values of test substances changed among algal species in the concentration ranges of 2.6–44; 12.2–65.8 and 86.8–132.9 mg/l for POEA, ROUNDUP CLASSIC and glyphosate, respectively. For MEDALLON PREMIUM and its components, no consistent toxicity order determined for green algae species examined. Toxicity order of ROUNDUP CLASSIC determined by chlorophyll content for *D. subspicatus* were: POEA (72h EC_{50} = 4.9±0.7 mg/l) > herbicide formulation (72h EC_{50} = 13.4±4.7 mg/l) > glyphosate active substance (72h EC_{50} = 73.8±6.4 mg/l). For MEDALLON PREMIUM the order was different: alkyl polyglucoside surfactant (72h EC_{50} = 50.9±0.7 mg/l) > active substance (72h EC_{50} = 73.8±6.4 mg/l) > herbicide formulation (72h EC_{50} = 157.2±60.6 mg/l). Significant differences were observed between 72h EC_{50} values determined by measurement of OD and chlorophyll content. Significant differences were observed in the sensitivity among the 3 green algae species. Glyphosate was more toxic to blue (72h EC_{50} = 27.1±7.74 mg/l) than for green algae.

Effects of glyphosate, ROUNDUP CLASSIC and POEA on the photosynthetic activity of *P. subcapitata* test organism were investigated by FluoroMeter Module (FMM) based on the chlorophyll-a fluorescence induction, where Fv/Fp value characterizing the photochemical efficiency of the PSII photochemical system and vitality index (RfD) of photosynthetic activity were determined. As a result of glyphosate treatments, at the lowest concentration (13.6 mg/l), an increase in Fv/Fp was observed, which can be explained by the hormeosis effect observed in algal reproduction inhibition tests. The concentration of glyphosate above 108.9 mg/l resulted in a significant reduction in the photochemical efficiency of the PSII photochemical system. Similarly to the Fv/Fp value, above 108.9 mg/l, a significant decrease was noted for RfD. Above 11.6 mg/l a significant decrease in the vitality index was observed. POEA did not influence the Fv/Fp values in the treated algae, while at the highest tested concentration (19.2 mg/l), a significant decrease in RfD was observed.

Biological effects were also tested on algal communities in biofilms. The aim of this study was to investigate and compare the dissipation of glyphosate in pure IPA salt and formulated forms (ROUNDUP CLASSIC herbicide formulation) in freshwater samples originated from Lake Balaton and River Danube, with and without the presence of natural freshwater biofilms. Dissipation was investigated as the biodegradation of glyphosate by microbial activities and physical sorption on the surface of biofilms and solid particles of water samples. Effects on algal communities (biomass, composition of green, blue and diatom algae) were also determined. Natural biofilms were grown on glass substrates fixed on AKK-1 type carrier buoys. The buoys were immersed in Lake Balaton and River Danube at fixed locations for six weeks to allow sufficient growth of biofilms. Bacterial biofilm formation was sampled daily for one week and on day 14 upon outplacement of the buoys. After the six-week colonization period, the buoys were moved into the laboratory, and the glass substrates were placed into 15 l glass aquaria. Water in the aquaria was changed every week with water obtained from the same locations, where the buoys had been located previously. Glyphosate IPA salt and ROUNDUP CLASSIC treated aquaria were spiked with 100 µg/l of glyphosate IPA salt and equivalent concentration of formulation upon each weekly water exchange. The kinetics of dissipation of glyphosate, measured by HPLC combined with UV-VIS absorbance detection or tandem mass spectrometry. The quantity and the biofilm structure of algal biomass were determined by with bbe Moldaenke BenthoTorch algae torch instrument based on real-time measurement of benthic algal concentrations by *in situ* quantification of chlorophyll-a fluorescence, *in vivo* fluorescence of algal cells, and scanning electron microscopy.

Significantly higher initial concentrations were measured 30 min after the addition of 100 µg/l of the glyphosate IPA salt in water samples originated from River Danube for formulated glyphosate treatment (101.4±6.2 µg/l), than with glyphosate alone (79.9±6.6 µg/l) due to the presence of formulating agent POEA, and dissipation to a residual level (57.6±1.4 µg/l) consequently took longer (approximately by 1 day). A possible mechanism involved in this process can be that the surfactant suppressed the physical adsorption of glyphosate on the solid–liquid surfaces (e.g. glass materials of aquaria, solid phase and floating particles in water samples). Degradation of glyphosate from its initial level (91.2±5.9 µg/l) was not detected in water samples from Lake Balaton, the level of glyphosate stagnated at 90 and 100 µg/l in case of the pure and POEA-formulated active ingredient, respectively.

In River Danube treated aquaria containing biofilms the phytotoxic effects of glyphosate, particularly if enhanced by a formulating agent, may have contributed to the observed decrease of the algal biomass relative to the untreated control. Moreover, the gradual increase in glyphosate concentrations detected after repeated weekly addition of 100 µg/l of pure glyphosate IPA salt is likely to be due to saturation of the sorption sites in the EPS matrix in the biofilm. By the fourth week, the total biomass increased, accompanied by significant decreases in glyphosate concentration, possibly due to the utilization of glyphosate from water as a nutrient by tolerant algal species (*Fig. 5* A). When glyphosate was applied in a formulated form, the treatment resulted in a rapid gradual decrease of the concentration of glyphosate during the first week in the presence of high biomass. The treatment resulted in a decrease in the algal biomass, relative to the untreated control, within two weeks. Possible factors contributing to this trend are the phytotoxic effect of the formulation and the increased production of the EPS matrix observed in a qualitative estimation based on the SEM images. The measured level of glyphosate was stagnant upon weekly additions of glyphosate. From the third week on, increasing glyphosate concentrations were detected likely due to the saturation of the sorption sites in the EPS matrix (*Fig. 5* B). Similarly to pure glyphosate treatment, the biomass increased by the fourth week. Despite the lower bioavailability of glyphosate in water, tolerant algal species occurred to be able to utilize glyphosate as a nutrient from the EPS matrix.

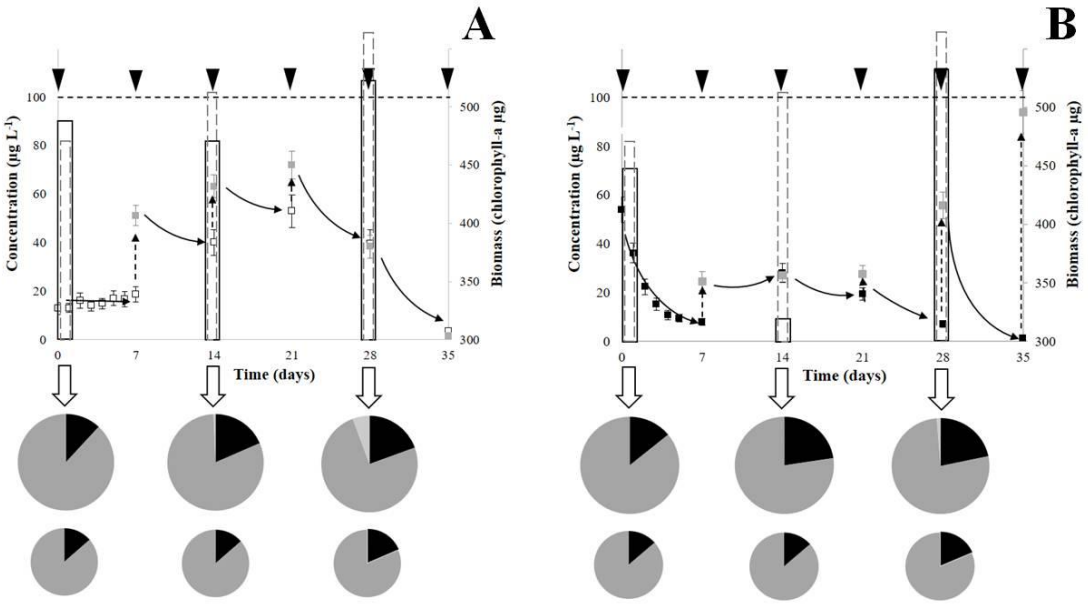

Figure 5. Dissipation of glyphosate (**A**) ROUNDUP CLASSIC (**B**) in water from river Danube in the presence of biofilms. Measured concentrations of glyphosate in pure (□) or formulated form (■) in water from initial glyphosate concentrations (■) in 30 min after each repeated glyphosate addition (▼). Arrows indicate concentration changes due to dissipation (*solid lines*) or reagent addition (*dashed lines*). Biomass levels in the treatment group (open black columns with solid line) and the untreated control (open grey columns with dashed line) are indicated. Corresponding algal composition (pie diagrams below each column, treatment group in the upper and control in the lower row) show the biomass proportion of cyanobacteria (*dark grey*), diatom (*black*) and green (*light grey*) algae.

Exposure of biofilms formed in Lake Balaton resulted in different dissipation patterns of glyphosate than those seen for River Danube. The phytotoxic effect of glyphosate or herbicide formulation resulted in a continuous decrease in the biomass during the five-week experimental period. Compared to the degradation without the presence of biofilms, lower concentrations of glyphosate were detected in the first week possibly attributed to chelate or complex formation with the EPS matrix. After the first week during the weekly, repeated addition of pure glyphosate into the aquaria, the concentration of glyphosate stabilized at the same level as observed in the first week, but on the fifth week the concentration (62.3 µg/l) of the spiked glyphosate dose was significantly reduced 30 min after the addition. Upon treatment with POEA-formulated glyphosate, the initial decline in glyphosate concentration during the first week was less rapid as observed with pure glyphosate. Upon repeated addition of formulated glyphosate, the entire dose (100 µg/l of pure glyphosate IPA salt – equivalent to 74.1 µg/l glyphosate acid) applied was detected in the water samples 30 min after treatment until the fourth week, when the level of glyphosate detected slightly dropped (86.5 µg/l). This is expected to result from an increased stress response of algal community to the exposure to ROUNDUP CLASSIC, potentially resulting in an increased EPS matrix production.

SEM analysis indicated considerable changes in biofilm structure. Realignment of the biofilms was typical, and glyphosate-sensitive species were replaced by tolerant ones like filamentous green algal species. The realignment of biofilms and the effects of glyphosate on the microbial community structure in freshwater were observed in other studies as well. The electron microscopic analysis also indicated increased production of the EPS matrix, relative to the corresponding negative controls, in each treatment group. Visual analysis of the SEM

images suggested an intensive EPS production for exposure to POEA-formulated glyphosate (*Fig. 6*). This phenomenon can be attributed to the protective mechanism of bacteria and algae to eliminate and reduce the effects of contaminants. Additionally, glyphosate can affect the metabolic processes of bacteria and algae simultaneously, resulting in an enhanced production of the EPS matrix as response to physical, chemical and biological stress factors.

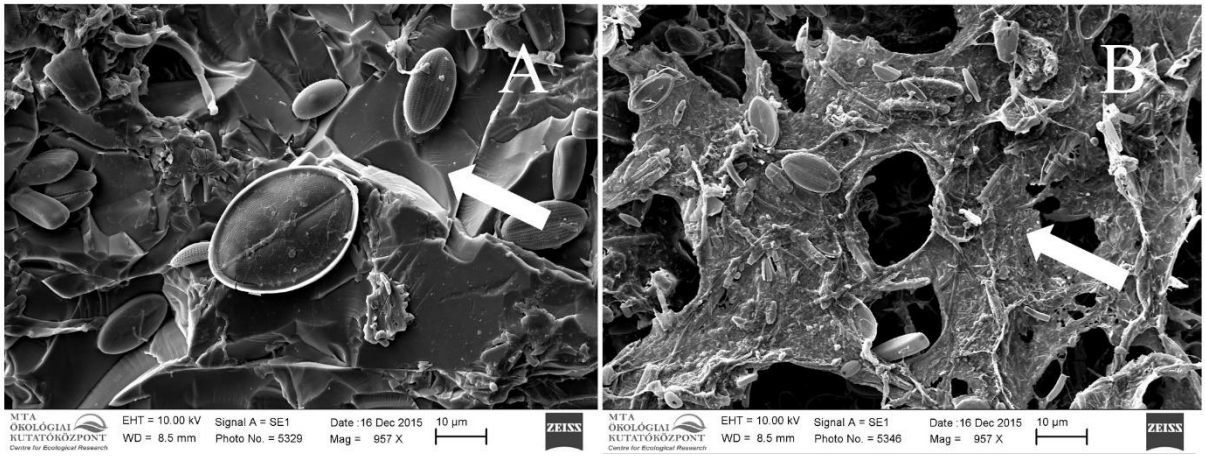

Figure 6. Increased production of EPS matrix (*indicated by arrow*) in natural biofilms from River Danube, due to treatment, visualized by scanning electron microscopy. (**A**) Control biofilm with smooth EPS layer. (**B**) Intensive EPS formation upon exposure to POEA-formulated glyphosate-based herbicide.

Klátyik *et al.* (2017) Dissipation of the herbicide active ingredient glyphosate in natural water samples in the presence of biofilms. *Int. J. Envir. Anal. Chem.*, **97** (10): 901-921.

Gyurcsó *et al.* (2017) Effects of glyphosate-based herbicides and their components on the embryonal development of zebrafish (*Danio rerio*): Assessment of the role of retinoids. *ISAEP 2017*. poster.

3.2.2. Cytotoxicity assays on human/mammalian cell lines

Cellular events including effects on cell viability, apoptosis, cell cycle and barrier function were studied in detail in the participating research institutes within the project. Cytotoxicity of glyphosate IPA salt, ROUNDUP CLASSIC and POEA were examined on NE-4C neuroectodermal (ATCC: CRL-2925) and MC3T3-E1 preosteoblast (93021013 Sigma Aldrich) cell lines. Effects on viability, apoptosis, DNA-damage, caspase 3/7 activity and cell cycle were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and Muse Cell Analyzer flow cytometer (Merck Millipore, Budapest, Hungary) using the proper kit suggested by the company.

MTT assay, as a colorimetric assay to measure cellular growth and survival of any cultured cell line, was carried out to measure cell viability in a biochemical (enzyme) assay for each substance. ROUNDUP CLASSIC markedly decreased NE-4C cell viability at concentrations above 0.0032%, with a substantial effect detected after 2 h, which significantly increased by 6 h, but no further inhibition was detected at 24 h. POEA inhibited cellular metabolism above concentrations of 0.000775 g/l (corresponding to a 0.0005% ROUNDUP CLASSIC) upon even 2 and 6 h exposure. Glyphosate IPA salt caused lower inhibition of the cell viability above 0.243 g/l (corresponding to a 0.05% ROUNDUP CLASSIC). After 24 h exposure IC_{50} values on NE-4C determined from sigmoid dose-response curves, were found to be 0.0036±0.0002%, 0.00375± 0.00045% and 0.3902±0.0319% for POEA, ROUNDUP CLASSIC and glyphosate IPA salt, respectively. The corresponding IC_{50} values for MC3T3-E1 blast cells were 0.00117±0.00022%, 0.0110±0.0009% and 0.548±0.130% (*Fig.7*).

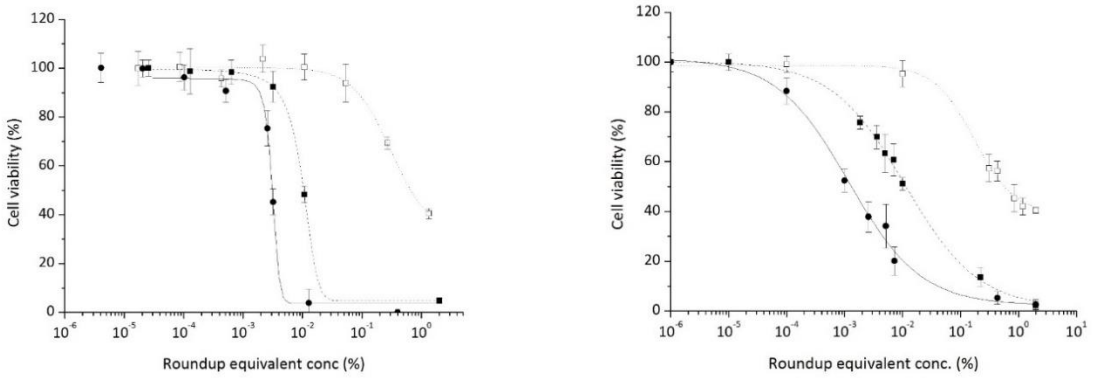

Figure 7. Concentration-dependent effect of exposure with glyphosate IPA (□), ROUNDUP CLASSIC (■) and POEA (●) after 24 h treatment on NE-4C (*left*) and MC3T3-E1 (*right*) cell viability determined by the MTT-assay.

The Muse Annexin V dead cell kit was used to determine the ratio of total apoptotic cells, after 24 h of exposure. IC_{50} value on NE-4C was 0.00092±0.00005%, 0.00238±0.00028% and 0.246±0.013 for POEA, ROUNDUP CLASSIC and glyphosate IPA, respectively in equivalents of diluted ROUNDUP CLASSIC. High level of apoptotic cells detected for POEA, which is 267-fold higher than for glyphosate IPA, and 2.6-fold higher than ROUNDUP CLASSIC. For MC3T3-E1 the corresponding values were 0.0117±0.0048%, 0.0167±0.0013% and 0.2731±0.0452%. DNA damage was determined via measurement of ATM and H2AX activation. For glyphosate IPA, ROUNDUP CLASSIC and POEA the highest rate of damage was observed at 0.13%, 0.001% and 0.00015% concentration, respectively.

The distribution of cells within a cell population among different phases of the cell proliferation cycle is an informative indicator, whether cell division of the population has been affected upon exposure to the test substances. The proportion of cells in the beginning DNA replicating (S), cell division (G2/M) and growth (G0/G1) phases was detected by the Muse Cell Cycle Assay Kit (MCH100106, Merck Millipore), utilizing propidium iodide-based staining of the DNA content of the cells showing a characteristic increase during DNA replication and subsequent decreases relative to cell size also detected, as seen in the DNA content index and histogram, as well as cell size index determined by the assay. After 24 hrs, for all test compounds ratio of cells in S phase decreased. After 48 hrs exposition this decrease was more outstanding, moreover ratio of cells in G0/G1 phase increased compared to control.

Cell integrity was visualized by holographic transmission microscopy, a novel, label-free, non-invasive, nondestructive and non-phototoxic method allowing both qualitative and quantitative measurements of living cells over time. In holographic microscopy the illuminating light is split into an object beam and a reference beam. The object beam upon illumination of the object is re-joined and interfered with the reference beam creating a hologram. Focusing within the hologram is possible to any point without any mechanical movement by iteratively created images any time after the actual recording. Cell morphology parameters, determined by holographic microscopy, are useful descriptors of cell viability and ongoing cell-morphological changes including the processes of cell differentiation, cell growth and cell death. To further assess how treatments with glyphosate, POEA and ROUNDUP affect cell motility, the sensitivity of NE-4C cells to these substances was characterized by real-time holographic imaging. Cells under toxic effects take rounded shape due to cytoskeletal response, and become detached from surfaces they had adhered to. A time-dependent decrease in cell area and an increase in maximum thickness of the NE-4C cells due to treatment were seen. The effect of glyphosate (0.0042%), POEA (0.0016%) and ROUNDUP (0.01%) on average cell area is seen on *Fig. 8*.

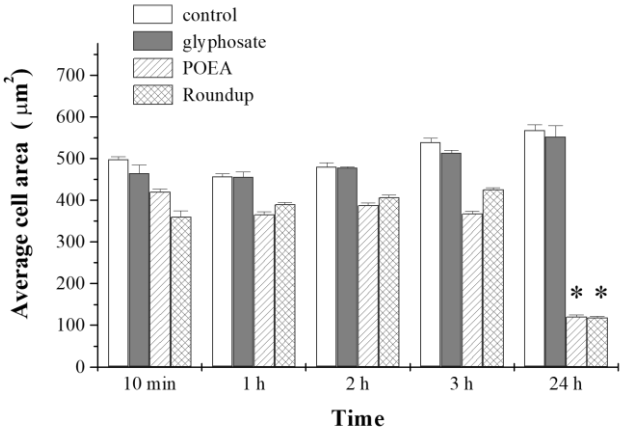

Figure 8. Time dependence of cell areas detected in holographic microscopy upon toxicant administration. ROUNDUP, glyphosate and POEA were applied at concentrations of 0.01%, 0.0042% and 0.0016%, respectively (concentrations corresponding to 0.01% ROUNDUP solution). ROUNDUP and POEA caused extensive cell death upon 24 hours of exposure (indicated by * mark).

The toxic effect of POEA and ROUNDUP CLASSIC was seen as rapidly as in 10 minutes, followed for up to 24 hours, while glyphosate did not cause statistically significant difference in average cell area compared to the control. Average cell area showed an increase in 24 hours in the control due to cell adhesion, while it was rapidly decreasing due to extensive cell death upon the effect of POEA or ROUNDUP, practically equitoxic with each other at concentrations 20-fold below agricultural application (*Fig. 9*).

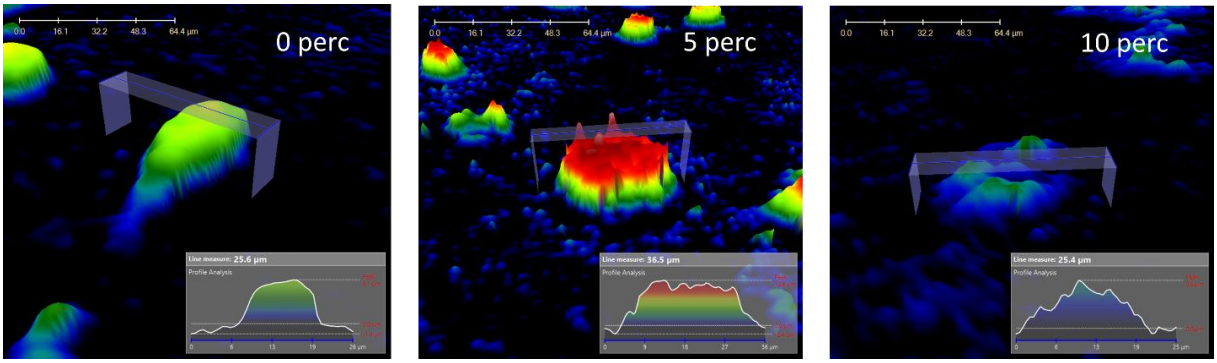

Figure 9. Time-dependent morphological changes of NE-4C cells exposed to ROUNDUP (0.1%), detected by phase contrast holographic microscopy. Images were captured every five minutes from the beginning of treatment with ROUNDUP (0.1%). After a few minutes of treatment the cells become round, then turn uneven and later break apart.

Cytotoxic effects of glyphosate IPA salt, 6 glyphosate-based herbicides (ROUNDUP CLASSIC, ROUNDUP WEATHERMAX, GLYFOS, KAPAZIN, TOTAL, MEDALLON PREMIUM) and 4 co-formulants frequently applied in glyphosate-based herbicides (POEA, POE alkyl phosphate ester, alkyl polyglucoside, quaternary ammonium compound) on placenta choricarcinoma (JEG3) cell line (ECACC 92120308). Cytotoxic effects were determined by the tetrazolium dye MTT assay. All co-formulants and formulations were comparably cytotoxic well below the agricultural dilution of 1% (18–2000 times for co-formulants, 8–141 times for formulations), and not the declared active ingredient glyphosate alone (*Fig. 10*).

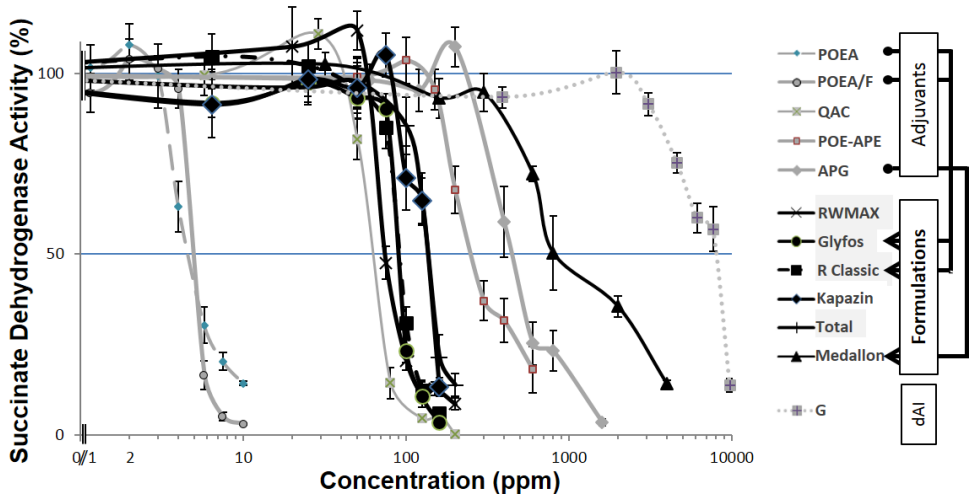

Figure 10. Dose-dependent cytotoxic effects of various glyphosate-based herbicide formulations, co-formulants and active ingredient glyphosate on JEG3 human placenta choricarcinoma cell line.

Individual and combined effects of glyphosate and POEA were also tested on cell barrier functions. Thus, paracellular permeability of IPEC-J2 cells in unilayer on polyester membrane inserts were determined by TEER, as a sensitive and reliable *in vitro* barrier model system to confirm the integrity and permeability of the monolayer, and fluorescein isothiocyanate-dextran (FD4) level in the basolateral region of the cells (see 3.1.2 before). After 2-hr exposition, no significant changes in TEER values were determined for glyphosate at 200x dilution compared to control, however, after 24-hr exposition a slight but significant increase was detected for the active ingredient. 2-hr exposition of POEA and ROUNDUP CLASSIC formulation increased the paracellular integrity to minimum level at 5000x and 2000x dilution, respectively. Measurement of FD4 level in basolateral region showed the same result. POEA and the herbicide formulation resulted significant decrease in FD4 levels, however, effects of POEA were 2-fold higher than ROUNDUP CLASSIC. Effects of POEA were further investigated to determine the concentration that caused no changes in integrity. POEA was investigated at dilutions of 200,000x, 2,000,000x, 20,000,000x and 200,000,000x. After 2-hr exposition no significant differences were detected in TEER values compared to control group. After 24-hr TEER values were higher for every concentrations of POEA than for control. Concentration of fluorescein isothiocyanate-dextran (FD4) was significantly higher in 200,000x dilution of POEA, however further dilutions caused no difference in FD4 values.

Reactive oxygen species (ROS) were determined with characterization of intracellular redox status by DCFH-DA and with measurement extracellular H_2O_2 changes by Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Glyphosate caused significant increase while POEA and ROUNDUP CLASSIC significant decrease in extracellular ROS levels at the concentrations investigated in TER and FD4 measurements. Determination of intracellular redox status triggered the same results. Decrease in extra- and intracellular ROS levels after POEA and ROUNDUP CLASSIC may be due to cell death.

As related to the assessment of glyphosate and its formulated herbicides being classified as a possible carcinogen, we also carried out studies on mammary carcinoma cells at the National Institute of Oncology. Our question was how glyphosate-based herbicides, their active ingredient glyphosate (revealed as a chemical chelator) and formulants influence the growing potential of mammary carcinomas and other sensitive cell types. Our specific aims were to

define the effects of glyphosate and its formulating surfactant on various, intensively growing cell types at cellular and molecular levels. For studying sensitivity, we used MTT based cell proliferation assay that enables measuring only living cells and can be read on a scanning multi-well spectrophotometer. Two types of breast carcinoma cell lines: the MCF7 invasive ductal breast carcinoma (estrogen receptor positive, progesterone receptor negative, no ERBB2 amplification) and the MDA-MD-231 invasive ductal breast carcinoma (estrogen receptor negative, progesterone receptor negative, no ERBB2 amplification) and other sensitive cells and a control cell line (HEK-293, originally derived from human embryonic kidney cells, widely used in cell biology research because of their reliable growth and propensity for transfection) were cultured under standard conditions. ROUNDUP CLASSIC and its detergent POEA were tested in 2% 50x diluted and 0.32% 300x diluted forms, respectively, while glyphosate (as IPA salt) (62%) was 46.3x diluted. Optical density measurements after MTT cell proliferation assay, indicating the high *in vitro* anti-proliferative effects of ROUNDUP and POEA in considerable concentrations. The effect of glyphosate on MCF7 and MDA-MD 231 breast cancer cells was less pronounced, in comparison to the compounds. A difference was found in the sensitivity of the MCF7 (ER hormone receptor positive) and in the MDA-MD-231 (ER hormone receptor negative) cells.

Detection of tumor associated antigens as cell surface structures that play important role in cell proliferation was carried out in formaldehyde-fixed paraffin embedded tissue sections. Immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded cancerous tissue sections with anti-GD3 monoclonal antibody and supersensitive TM One Step Polymer IHC Detection Kit System (BioGenex), using 3,3-diaminobenzidine (DAB) substrate Kit. Primary invasive ductal breast carcinoma cell cultures were applied in chamber slide conditions. The immunofluorescence assay, performed with specific monoclonal antibodies and confocal laser microscopy as detection system, was used efficiently o follow even mild changes on the cell surface at membrane antigen expression level. Due to our major interest and the great importance of glycolipid-based membrane structures in the cell membrane, we focused on the expression on disialylated glycosphingolipides. We used an indirect immunofluorescence assay to label the cells that were grown to confluent in the chamber microcultures and underwent treatments with glyphosate, ROUNDUP or POEA at potentially effective concentrations based on the previous results obtained by the MTT proliferation assay. In the chamber slide technique cells may be observed at any point during culture using an inverted light microscope. Additionally, treated and control cells can be labeled by a two-step indirect immunofluorescence assay *in situ*, without the need to detach cells enzymatically or mechanically from the surface, and cause any damage to the cells. Upon fixation with 4% paraformaldehyde in phosphate buffered saline (PBS) and blocking wells with 3% bovine serum albumin (BSA) first (disialylated glycosphingolipid-specific) and second (fluorescein isothiocyanate labeled goat anti mouse IgG (F(ab')2 fragment) antibodies were added, and immunofluorescence labeling was detected by confocal laser microscopy and also by conventional immunofluorescence microscopy that visualized membrane structure changes due to the treatment, directly. We found with **i**ndirect immunofluorescence FACS analysis, that MCF7 breast cancer cells as well as MDA-MD 231 express disialylated glycospingolipids, GD3 ganglioside extensively. Based on the importance of these glycosphingolipids (cell proliferation, adhesion, signal transfer, apoptosis), the measurable effects of the treatments indicated that POEA diminished the GD3 ganglioside expression on MCF7 cells: the intensity of monoclonal antibody binding and so the immunofluorescence labeling was strongly reduced according to the immunofluorescence microscopy images.

Cellular events were also followed by expression analysis of cellular markers by RT-PCR. MCF7 and MDA-MD 231 breast carcinoma cells treated with glyphosate, ROUNDUP CLASSIC and POEA were harvested after the defined treatment periods. Control and treated

cells were centrifuged, washed twice carefully with sterile PBS, and cell suspensions were pelleted in RNAse-free Eppendorf tubes. Cell pellets were suspended in suitable volumes of Trizol, adjusted to the counted cell numbers and stored frozen at -70°C. Total RNA isolation was performed using the Nucleospin RNA kit. In the RNA kit with column technique, DNase treatment was included to eliminate potential genomic DNA contamination using a NanoDrop ND-1000 spectrophotometer and RNA Spike kit (RNA 6000 Nano), RNA Nano Chip (On-Chip-Electrophoresis) with an Agilent 2100 Bioanalyzer microfluid technology with automatic gel electrophoresis and highly sensitive detection by electronic microcapillary LabChips. Samples with 200 ng pure RNA were stored under suitable conditions until gene expression analysis with Real Time PCR. Reduced proliferation was observed in the MDA MD-231 and MCF7 cell lines. The effect was different in ER-positive and -negative cells, and a lower tumor-associated glycosphingolipid expression could be detected by indirect immunofluorescence assay and fluorescence microscopy. The expression change of GD3 gangliosides, highly tumor-associated antigens was especially remarkable. First-line gene expression analysis and Qiagen ingenuity pathway analysis revealed different gene expression patterns upon exposure to ROUNDUP, POEA or glyphosate, also shown in gene expression regulation in the two breast cancer cell lines, indicating the involvement of GD3 synthase gene machinery. Our data show a new aspect of the glyphosate and ROUNDUP treatments of various cells, emphasizing the cell membrane integrity damaging potential of these xenobiotics *via* glycosphingolipid-containing lipid rafts. The characteristic transcriptional changes found by gene expression analysis support the diminished GD3 disialylated glycosphingolipide expression found by immunofluorescence microscopy. The study provides important information on the proliferation inhibition and apoptosis promoting effects of adjuvants in glyphosate formulations.

Székács *et al.* (2014) Environmental and toxicological impacts of glyphosate with its formulating adjuvant. *Intl. J. Biol. Biomol. Agric. Food Biotech. Engineer.*, **8** (3): 219-224.

Székács *et al.* (2016) Label-free optical biosensors for monitoring cellular processes and cytotoxic agents at interfaces using guided modes and advanced phase-contrast imaging techniques. In: Biosensors for Security and Bioterrorism Applications (Nikolelis, D. P., Nikoleli, G.-P., Eds.), Springer, Cham, Switzerland, pp. 443-468. doi: 10.1007/978-3-319-28926-7

Defarge *et al.* (2016) Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *Int. J Environ. Res. Pub. Health*, **13**: 264.

Farkas *et al.* (2018) Label-free optical biosensor for real-time monitoring the cytotoxicity of xenobiotics: a proof of principle study on glyphosate. *Hazard. Mat.*, submitted.

Kotlan *et al.* (2018) Reduced tumor-associated glycosphingolipids in cell membrane lipid rafts and a diminished proliferation rate in mammary breast carcinoma cultures after incubation with glyphosate-based formulations. *J. Immunother. Cancer*, submitted

Studies also targeted on genotoxicity, hormonal modulation and effects on gender development. Endocrine-disrupting effects of glyphosate IPA salt, six glyphosate-based herbicides (ROUNDUP CLASSIC, ROUNDUP WEATHERMax, GLYFOS, KAPAZIN, TOTAL, MEDALLON PREMIUM) and four co-formulants frequently applied in glyphosate-based herbicides (POEA, POE alkyl phosphate ester, alkyl polyglucoside, quaternary ammonium compound) on placenta choricarcinoma (JEG3) cell line (ECACC 92120308). All these compounds were measured on aromatase activity, a key enzyme in the balance of sex hormones, below the toxicity threshold. Aromatase inhibition was tested at concentrations between 1.2–3 times below the no observed effect concentration (NOEC) and 1.2–2 times below the toxic level, in order to avoid measuring the general toxicological effect. Aromatase assays were performed at 25 mg/l of GLYFOS (containing 2.5 and 16 mg/l of POEA and glyphosate, respectively), and 300 mg/l of MEDALLON PREMIUM (containing 120 mg/l of APG, its co-formulant, and 190 mg/l of glyphosate). Aromatase activity was decreased both by the co-formulants alone (POEA and alkyl polyglucoside) and by the formulations, from concentrations

800 times lower than the agricultural dilutions; while glyphosate exerted an effect only at 1/3 of the agricultural dilution (*Fig. 11*). It was demonstrated for the first time that endocrine disruption by GBH could not only be due to the declared active ingredient but also to co-formulants. These results could explain numerous in vivo results with glyphosate-based herbicides not seen with glyphosate alone; moreover, they challenge the relevance of the acceptable daily intake (ADI) value for herbicide exposures, currently calculated from toxicity tests of the declared active ingredient alone.

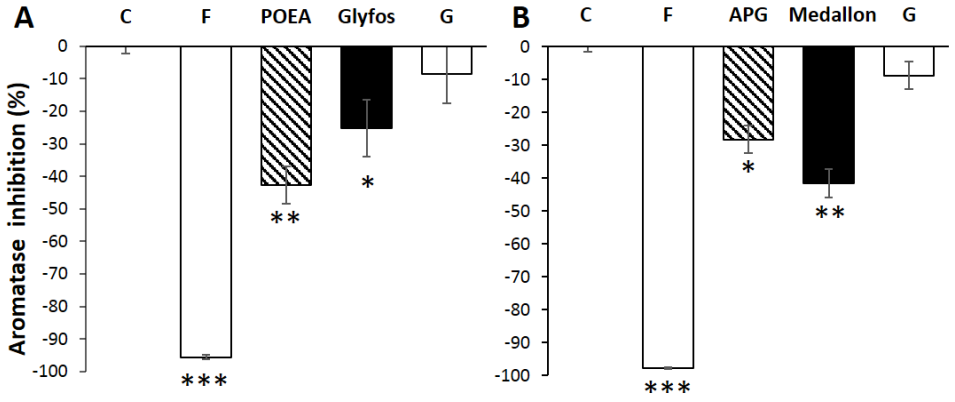

Figure 11. Aromatase inhibition by formulations and their adjuvants alone at similar levels.

Two other pesticide co-formulants, quaternary ammonium compound and POE alkyl phosphate ester, not declared in these formulations were studied, and they effectively inhibited aromatase by around 80%. This is, of course, under the NOEC (respectively 1.4 and 1.5 times below, and half of the toxicity threshold for each). This could indicate a common mechanism of co-formulant effects. When formulations are compared at the same dose far below agricultural dilutions (at 50 mg/l), the following hierarchy of endocrine disruption was observed: ROUNDUP WEATHERMAX > ROUNDUP CLASSIC > TOTAL > KAPAZIN > GLYFOS. Effects of the formulations on the endocrine system are not due to glyphosate active ingredient, as it is inefficient under these conditions.

Genotoxic effects of glyphosate, POEA and ROUNDUP CLASSIC were determined by the sensitive Comet assay, a fluorescent method for analysis of DNA damage and DNA repair mechanisms at individual cell level. Experiments were performed on human HepG2, NE-4C and MC3T3-E1 cell lines. Untreated cells were applied as negative control, 0.01% H_2O_2 as positive control. After 24-hr exposition, cells were transferred into Eppendorf tubes and mixed into agarose gel that was placed in the surface of an object slide. These embedded cells were lysed to disaggregate the membrane. Nuclei were electrophoresed, where under electrical circumstances DNA fragments effuse shaping the typical comet form detected by fluorescent microscope (Olympus IX73) after treatment with ethidium bromide. The DNA content of the cell and the tail, as well as the length of the head and the tail were determined by LUCIA Comet Assay 3.5 software. Based on these parameters tail moment values were calculated, as the product of the tail DNA content and the mean distance of migration in the tail.

ROUNDUP CLASSIC equivalent POEA (IC_{50}=0.0008±0.0005) were 443-fold and 87.5-fold more toxic than ROUNDUP CLASSIC equivalent active substance (IC_{50}=0.351±0.025) and ROUNDUP CLASSIC itself (IC_{50}=0.0700±0.0003), respectively on HepG2. ROUNDUP CLASSIC equivalent POEA (IC_{50}=0.00024±0.00006) were 1500-fold and 13-fold more toxic than ROUNDUP CLASSIC equivalent active substance (IC_{50}=0.355±0.045) and ROUNDUP CLASSIC itself, respectively on NE-4C. ROUNDUP CLASSIC equivalent POEA (IC_{50}=0.0019±0.0017) were

540-fold more toxic than ROUNDUP CLASSIC equivalent active substance (IC_{50}=1.057±0.072) on MC3T3-E1.

Cytotoxic and genotoxic effects of glyphosate, four glyphosate-based formulations (ROUNDUP, GLYFOS, TOTAL, MEDALLON) and seven formulating agents applied commercially in formulations (POEA, alky polyglucoside-APG, quaternary ammonium compound-QAC, sodium alkyl polyglucoside sulfosuccinate and citrate-SAPS and SAPC, POE alkyl phosphate ester-POE APE) were investigated by the SWITCH (*Salmonella* Weighting of Induced Toxicity Cyto/GenoTox for Human Health) test. SWITCH is a luminescent/fluorescent bioassay as a successor of the SOS-LUX test for rapid toxicity (genotoxicity and cytotoxicity) testing. It makes use of two sensing and reporting systems for the two biological endpoints under investigation: the SOS-LUX test and the LAC-Fluoro test. No compounds exerted genotoxic effects on the prokaryotic organism. The toxicity profile was the following (LC_{50} values in parentheses indicate the dilution rate of the given compounds): glyphosate (1.17%), MEDALLON (0.29%), POE APE (0.098%), QAC (0.007%), APG (0.007%), ROUNDUP (0.006%), TOTAL (0.005%), SAPC (0.004%), GLYFOS (0.004%), SAPS (0.002%) and POEA (0.0005%).

Endocrine disrupting effects of the components in ROUNDUP CLASSIC (glyphosate and POEA) were investigated *in vivo* on zebrafish (*D. rerio*). At the beginning of the test 2 weeks old fish juveniles were placed in 10 l aquaria. Ethinylestradiol (EE), as a positive control was determined at 5 ng/l, glyphosate at 100 µg/l and POEA at 37 µg/l. Untreated control was prepared in dechlorinated tap water. For EE, glyphosate and POEA, exposition time for fish in Group A was 8 weeks and for Group B 20 weeks. Fish in Group A after 8 week were placed in freshwater aquaria for 12 weeks. Sex determination was performed based on morphological characteristics and by histological evaluation. Blood samples were collected for Tunel assay (Group B) and DiOC6 staining (Groups A and B). Cell determinations were performed by flow cytometry, sorting and histology. After the 20-week experiment the remaining male and female zebrafish were put into a spawning aquarium and after the spawning, the collected eggs were further tested by using the OECD 236 guideline „*Fish Embryo Toxicity Test* (FET test)". Concentration of glyphosate in treated aquaria was measured weekly by HPLC method developed in the frame of this project, and the mean glyphosate concentration in the water of glyphosate-treated aquaria during the 24 weeks of treatment was found to be 66.6±13.7 ug/l.

There were no hermaphroditic zebrafish present in either treatment groups. The ratio of female/male fish was significantly higher in EE treated aquaria, and the number of females was also higher in glyphosate and POEA treatments. Differences compared to control was more pronounced in Group B, after 20-week exposition (*Fig. 12*).

The number of Tunel+ cells in the whole blood samples of control zebrafish was 244.0±151.4, while the corresponding value in the EE treatment was 202.9±79.6. Fish in glyphosate- and POEA-treated groups had 359.6±303.9 and 349.8±248.9 Tunel+ cells in their blood samples, respectively. EE-exposure caused a slight decrease in the number of Tunel+ cells compared to controls. Glyphosate and POEA exposures caused an increase in the numbers of Tunel+ cells compared to control, but the differences were statistically not significant.

The number of granulocyte (R1 population) of the male zebrafish in Group B after EE-exposition was 511±309.3 that was significantly lower compared to control (p=0,022). In glyphosate- and POEA-treatments numbers of granulocyte were 488.92±360.15 and 887.38±1063.4, respectively. Glyphosate, similarly to EE significantly decreased the number of cells in whole blood samples (*Fig. 13* R1). The size of erythrocyte (R2) population in control was 21293±2031 EE exposition resulted in a significantly higher cell numbers with 23469±592 value (p=0,015). Cell numbers is glyphosate (22572±1304) and POEA (22211±2086) treatment did not differ significantly from the control and EE (*Fig. 13* R2). The size of erythrocyte (R3)

population for EE exposition was 447.08±240.06 that was not significantly different from control (605.0±392.8). The R3 population of glyphosate- and POEA-exposed male zebrafish was 989.2±645.4 and 680.5±411.9, respectively (*Fig. 13* R3). The lymphocyte (R5) population of female zebrafish in Group B after EE exposition was 4880.7±662.3. The corresponding values were 4366.2±435.7, 4093.3±513.1 and 4549.1±610.8 for glyphosate, POEA and control, respectively (*Fig. 13* R5).

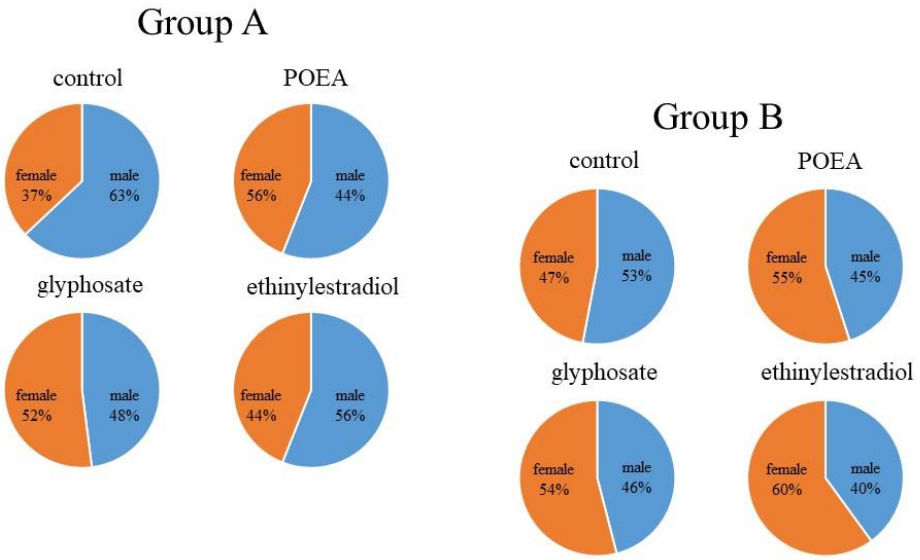

Figure 12. Ratio of female and male fish after 8-week exposition followed by 12-week control condition (Group A) and after 20-week exposition (Group B).

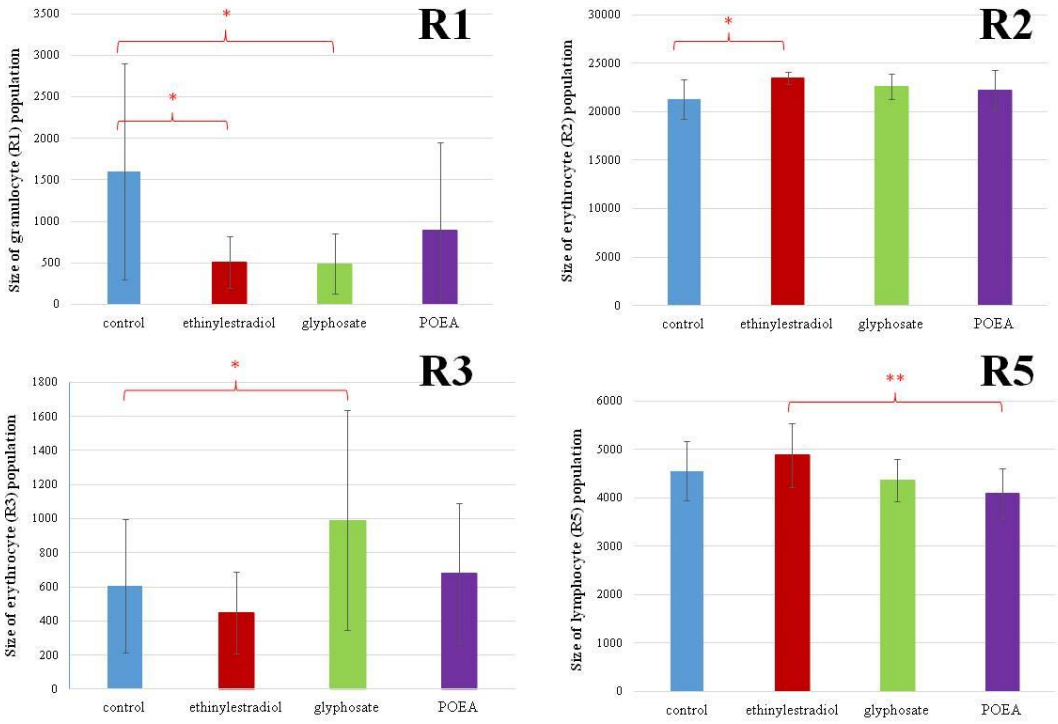

Figure 13. Size of granulocyte populations (R1, R2, R3, R5) in male zebrafish from Group B treated with glyphosate, POEA, ethinylestradiol (positive control) and untreated (negative control) after 20-week exposure. Significant differences are marked by red stars.

4. Aspects of risk assessment/authorization and application

4.1. Comparative evaluation of authorization of VDs and PPPs

Authorization and distribution of agrochemicals are strictly regulated in the EU. Important similarity aspects include the legal approval systems being focused on scientific evidence-based risk assessment (RA) and putting a strong emphasis on safety, primarily toward improving human health. Possible direct or indirect environmental risks have received increasing attention lately in both groups; yet regulatory pharmacology and toxicology of VDs are more pronouncedly oriented by a comparative medicine aspect, then the assessment of PPPs. Extensive control of VDs is required in the EU, and thus, the requirements are very strict not only for quality and efficacy but also for safety, including animal and human health and environmental risk assessment (ERA), similarly to the assessment and regulation of human medicines. Upon revision, veterinary legislation Directives 81/851/EEC and 81/852/EEC were amended by Directives (EEC) 2004/28 and 2009/9. Specific directives and legal specifications regulate the distribution and required quality of veterinary substances, including veterinary medical products, ready-made veterinary products, blood products, and homeopathic preparations. The conditions of marketing authorizations for medicinal products for human and veterinary use are set by Regulation (EC) 712/2012 amending Regulation (EC) 1234/2008. In the EU, two main processes are available for authorizing veterinary medicines: a centralized EU procedure and national protocols, the former by the European Medicines Agency (EMA).

PPPs are governed in the EU by Regulation 1107/2009 (EC), the “Pesticide Act”. A rather important feature of the pesticide registration policy is that pesticide active ingredients are authorized at the EU level, while formulated plant protection products and their uses on given crop commodities are registered at Member States (MS) level, in accordance with the corresponding EU rules and regulations. Such a dual registration protocol has certain, clear benefits, e.g., the formulated products are approved according to regional needs (ecological considerations—biogeographical regions) and also results in disadvantages (e.g., regulatory rigidity as given problems with the formulated products may not be addressed at EU level, but have to be dealt with by each MS. In the process of authorization, active ingredients are evaluated in scientific evidence-based RA by the European Food Safety Authority (EFSA), established in 2002. RA statements issued by EFSA, debated, and commented by the MSs are the basis of the subsequent EC decisions regarding authorization. Authorization rely on the determination of physico-chemical, toxicological, and ecotoxicological properties of the substances (the active ingredient or its mixture with its adjuvants), and data determined are used in scientific evidence-based ERA on the basis of both the Pesticide Act and Regulation 1907/2006 (EC), the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) Act, supervised by the European Chemicals Agency (ECHA). Certain toxicity tests required to register plant protection products are often performed with the active ingredient alone, not with the pesticide formulation itself. Moreover, ingredients inert in the main effect of the preparation are generally not even indicated on product labels and are often claimed to be confidential business information. On the basis of the current legislation, substantially simpler ERA is sufficient for additives compared to the active ingredients. The conditions of the authorization and commercial distribution of surfactants (e.g., detergents) in the EU are set by Regulation 648/2004 (EC), but it focuses primarily on general-purpose surfactants used in laundry detergents and cleaning supplies. On the basis of the safeguard clause, if a given surfactant (e.g., detergent) is considered as a risk to human or animal health safety or to the environment by one of the MSs, temporarily special conditions or the proscription of the commercial distribution of the products containing the adverse component can be applied on the area of the given MS.

Klátyik *et al.* (2017) Authorization and toxicity of veterinary drugs and plant protection products: residues of the active ingredients in food and feed and toxicity problems related to adjuvants. *Front. Vet. Sci.*, **4**: 146.
Székács (2017) Environmental and ecological aspects in the overall assessment of bioeconomy. *J. Agric. Environ. Ethics*, **30** (1): 153-170.
Szekacs and Komives (2017) Research directions in plant protection chemistry. *Ecocycles*, **3** (2): 4-12.

4.2. Residues in the European food chain

To illustrate past and current trends of VD and PPP residues in food and feed in the European Union, we have completed a thematic analysis of corresponding data of the Rapid Alert System for Food and Feed (RASFF) as a background study for the current project. RASFF is the most effective system in the EU to report non-compliances in agricultural commodities and food products with food/feed safety regulations to ensure a direct and real-time exchange of information among EU Member States (MSs) and to assist sustenance of an outstanding food/feed safety status. The four most prevailing causes of notifications in RASFF are mycotoxins, pathogenic microorganisms, pesticide residues, and heavy metals. Residues of pharmaceuticals (human and veterinary combined) are ranked 7th among the causes of notifications. VD and PPP residues between 2002 and 2016 were 2,036 and 3,527, respectively, indicating more than 70% higher occurrence rate for pesticide residues. The highest proportion of non-compliances for VDs were recorded in the 2002–2005 period, while corresponding period for pesticide residues is 2011–2015. The difference became even more visible after 2012, when monitored data became subject to additional risk assessment (RA) in RASFF. While the proportion of the category of "uncertain severity" decreased below 20% shortly after the introduction of the additional RA in RASFF, it lengthily remained at 50% for pesticide residues, and this persisting tendency could be reversed only by 2016, also seen in the number of the documented cases. The initial high number of reported cases in 2002 for VD residues has successfully been pushed to a level below 100 cases annually by 2006. In contrast, the number of notification cases for pesticide residues shows a gradual increase from a low (approximately 50 cases annually) initial level until 2005, with a drop only in 2016, still representing over 250 cases annually.

The survey published in this subject has also summarized additives and surfactants used in VDs and PPPs. In addition to pointing out that registration system of VDs appears to be more efficient in eliminating residue problems in food and feed, the study has demonstrated the toxicity problem represented by lacking authorization regulations regarding additives and surfactants.

Klátyik *et al.* (2017) Authorization and toxicity of veterinary drugs and plant protection products: residues of the active ingredients in food and feed and toxicity problems related to adjuvants. *Front. Vet. Sci.*, **4**: 146.

4.3. Re-registration of glyphosate in the European Union

One of the most controversial societal issues today, regarding pesticide registration in the European Union (EU) may be the case surrounding re-registration of the active herbicide ingredient glyphosate. This issue is quite substantially related to agricultural surfactants, particularly POEA. Key concepts and challenges in the re-registration of the herbicide active ingredient glyphosate in the EU, with related environmental analytical and toxicological/ecotoxicological problems, have been discussed in detail in a corresponding review publication within the project.

Central problems in this issue have been (a) conflicting statements between international regulatory or assessment agencies regarding the carcinogenicity status of glyphosate; and (b)

severe toxicological concerns regarding adverse effects of the formulant POEA used in glyphosate-based herbicides. During the process by the European Food Safety Authority (EFSA) preparing the documentation for re-registration of glyphosate (on the basis of risk assessment by the German Federal Risk Assessment Institute, BfR) appeared the evaluation by the International Agency for Research on Cancer (IARC) classifying glyphosate into Group 2A, "probably carcinogenic to humans". This strong hazard statement divided scientific circles and official health and environmental authorities and organizations, even though EFSA and the European Chemicals Agency (ECHA) assessed glyphosate as "unlikely to pose a carcinogenic hazard to humans" and "not classified as a carcinogen", respectively. Surfactants played an important role in this debate, as formulated glyphosate products – especially with POEA and related compounds – have been shown to cause stronger cytotoxic or endocrine disrupting effects than the active ingredient glyphosate alone. The decision regarding re-registration of glyphosate has to consider both hazard-based (IARC) and risk-based analysis (EFSA); the former may not be suitable to calculate practical significances, and the latter being challenged if exposure estimations are uncertain in light of new data on residue levels. The results of current analytical surveys on surface water are particularly worrisome. In turn, the precautionary principle appears to be the optimal approach in this case for regulation in the European Union.

To our pride, our results obtained in the current project have been considered in the debate. We summarized the re-registration issue in an outcoming review article in the periodical *Frontiers in Environmental Science*.

Székács and Darvas (2018) Re-registration challenges of glyphosate in the European Union. *Front. Environ. Sci.*, accepted for publication, MS ID 313802

4.4. Practical application in spice paprika cultivation

Residues of active ingredients and formulating surfactants may exert environmental or human health risks when entering the food/feed commodity chain. Pesticides are a hazard factor obviously in crop production, within which environmental and food safety of spices, used for flavoring in food production, culinary, catering and households, receive relatively low attention, particularly in low volume spice trade networks, even though potential spice contaminants may exert adverse effects on food safety and quality. Contamination surveys of spices generally focus on microbial impurities or mycotoxins. Yet, pesticide residues are also prevalent chemical contaminants in spices of the *Capsicum* species, including chili and spice paprika. To assess environmental and food safety and to maintain the quality of the "Hungaricum" spice paprika products, the levels of pesticide residues as contaminants in spice paprika and chili were discussed on the basis of RASFF alerts and notifications, and on the basis of other contamination cases reported in the scientific literature. Currently, 51 pesticide active ingredients are registered in EU for paprika cultivation dominated by insecticides. herbs and spices occurred within the top 10 product categories of notifications by RASFF in 2014 (altogether 121 notifications during the year).

In our investigation of pesticide residues in various environmental and paprika fruit samples originating from intensively cultivated fields in the Southern region of Hungary, Bács-Kiskun County near Kalocsa, pesticide residues were not detected in the collected paprika samples. In the soil samples, tefluthrin, trifluralin* and DDT* (with decomposition products: DDD and DDE) and, in one case, chlorpyrifos contamination were detected; in some soil samples, atrazine*, diazinon* and, in one case, metolachlor* were determined, but were not quantified. In contrast, no detectable pesticide residues were found as soil contaminants from paprika cultivation fields managed under ecological (organic) farming. As a surface water contaminant, trifluralin* has been detected in 50% of the collected water samples. However, pesticide residues were not detected in the investigated paprika samples. A half of the soil

samples from intensive cultivation fields were contaminated with tefluthrin, which is one of the most hazardous pyrethroids applied by pest control technology in soils. Consequently, the residues of persistent compounds could long be detected in environmental matrices.

Spice paprika production is a complete process, from field to the packaged product, where the most important aim is to eliminate all contaminant (*i.e.* pesticide residues, microbes) from the fruits. Monitoring pesticides in expediently treated red pepper crop, ripened half-product and dried end-product spice paprika, in a model experiment, indicated characteristic changes in the residue levels of four active ingredients studied. Residue levels detected, especially for chlorpyrifos, correlated well with pesticide treatments at increasing dosages applied. Residues of chlorpyrifos found even in samples treated with low dose verify that the ban on the use of this active ingredient in red pepper crops was reasonable to avoid risks of contamination. Results also indicated contamination risks at high doses of active ingredients penconazole and cypermethrin. Characteristic changes in bioactive components (carotenoids and tocopherols) in the finished product spice paprika depend on pesticide treatments and dosages. Thus, our study indicates the effect of intensive pesticide treatment not only on the pesticide residue level occurring in the crop, but also in the deterioration of product composition.

Klátyik *et al.* (2017) Pesticide residues in spice paprika and their effects on environmental and food safety. *J. Food Nutr. Res.*, **56** (3): 201-218.

Székács *et al.* (2018) Environmental and food safety of spices and herbs along global food chains. *Food Control*, **83**: 1-6.

Mörtl *et al.* (2018) The effect of intensive chemical plant protection on the quality of spice paprika. *J. Food Comp. Anal.*, **67**: 141-148.

Klátyik *et al.* (2018) Quality management in spice paprika production: from cultivation to end product. In: Quality Management Systems (Kounis, L., Ed.), InTech. Rijeka, Croatia, accepted for publication

Summary

Agricultural surfactants are supposed to be inert additives, yet these substances commonly exert biological side-effects, in given cases synergistic with those of the active ingredients of these preparations. Surfactants used in veterinary and pesticide formulations enter the environment and create potential exposure to a number of non-target organisms. Research carried out in this project demonstrated altered toxicity of veterinary and even more pesticide formulations compared to their active ingredients alone; and identified biochemical and (eco)toxicological hazards regarding certain agricultural surfactants including cytotoxicity (on cell lines of epithelial, neural and other tissues, as well as stem cells and tumor cells), endocrine disrupting effects, as well as aquatic ecotoxicity. Thus, in *in vivo* biotests, on *D. magna* the formulated antimicrobial preparation SUMETROLIM was found to be more toxic (EC_{50}=106.2±54.9 mg/l) than its active ingredients sulfamethoxazole and trimethoprim, but such interaction did not affect its main activity on *Staphylococcus aureus* and *Escherichia coli*. Similarly, toxicity of the formulated neonicotinoid insecticide APACHE 50 WG on *D. magna* (EC_{50} = 11.4±3.7 mg/l) was 50 times higher than that of its active ingredient clothianidin, and glyphosate-based herbicides (ROUNDUP CLASSIC, MEDALLON PREMIUM) were shown to be 10-50 times more toxic than their active ingredient glyphosate, proven to be due to the formulating surfactants polyethoxylated tallow amine (POEA) and alkyl-polyglucoside. Similar *in vivo* effects were demonstrated on algae and on the zebrafish (*Danio rerio*). Such differential toxicity of glyphosate and its formulants has been shown in *in vitro* cytotoxicity assays on numerous cell lines, including NE-4C murine neuroectodermal tem cell-like, MC3T3-E1 murine preosteoblast, JEG3 placenta choricarcinoma, IPEC-J2 porcine intestinal epithelial, MCF7 and MDA-MD-231 human breast carcinoma, HepG2 human hepatocarcinoma and HEK-293 human embryonic kidney cell lines, and effects were shown to be due to enhanced apoptosis

through reactive oxygen species and DNA damage by immunofluorescence staining, single cell gel electrophoretic Comet assay, flow cytometric cell analysis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test, holographic microscopy and expression analysis of cellular markers by real time PCR and Qiagen ingenuity pathway analysis. Cell membrane integrity damaging effects *via* glycosphingolipid-containing lipid rafts were also evidenced, while the role of the retinoid pathway could not be shown due to analytical sensitivity limitations. Endocrine disrupting properties were shown by aromatase inhibition tests.

Results regarding the occurrence and effects of active ingredients and formulating surfactants not only broadened our knowledge in environmental analysis and toxicology, as clearly identified *in vitro* cytotoxicity and endocrine disrupting effects of various surfactants e.g., POEA, POE alkyl phosphate esters, alkyl polyglucosides, quaternary ammonium compounds, but also resulted in practical implications for risk assessment, regulatory approaches and environmental and food safety monitoring. As for regulatory measures, they documentedly contributed to the 2016 ban on the use of POEA in glyphosate-based herbicides in the EU.

Results obtained strongly support that this research should be extended. Possible research directions are diverse including, but not limited to, several topics: (a) broadening the range of surfactants included in the cyto- and ecotoxicity tests; (b) including other types of biotests to identify adverse effects and their molecular mechanisms; (c) updating current exposure assessment in light of the results of environmental and biomonitoring; (d) evaluating whether current regulatory measures are of sufficient severity e.g., POEA has been banned in glyphosate-based herbicides and not other formulations or technology applications; (e) possible harmonization between policies in the authorization of veterinary drugs and plant protection products to improve biological product safety in the latter group; (f) assessing possible consequences for ecological agriculture, whether formulated pesticides currently allowed in ecological farming should be reconsidered due to the formulation surfactant and additive content.

Publications, reports

Scientific reports, publications prepared in the scope of the project:

Scientific articles in periodicals with impact factor or referred by ISI:

Székács, A., Mörtl, M., Fekete, G., Fejes, Á., Darvas, B., Dombos, M., Szécsy, O., Anton, A. (2014) Monitoring and biological evaluation of surface water and soil micropollutants in Hungary. *Carpathian J. Earth Environ. Sci.,* **9** (3): 47-60. (IF: 0,630)

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Defarge, N., Takács, E., Lozano, V., Mesnage, R., Spiroux de Vendômois, J., Séralini, G.E., Székács, A. (2016) Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *Int. J Environ. Res. Pub. Health,* **13**: 264. (IF: 2,101)

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Győri, J., Farkas, A., Stolyar, O., Székács, A., Mörtl, M., Vehovszky, Á. (2017) Inhibitory effects of four neonicotinoid active ingredients on acetylcholine esterase activity. *Acta Biol. Hung.*, **68** (4): 345-357. (IF: 0,506)

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Takács, E., Klátyik, Sz., Mörtl, M., Rácz, G., Kovács, K., Darvas, B., Székács, A. (2017) Effects of neonicotinoid insecticide formulations and their components on *Daphnia magna* – the role of active ingredients and co-formulants. *Int. J. Envir. Anal. Chem.,* **97** (9): 885-900. (IF: 1,208)

Klátyik, Sz., Takács, E., Mörtl, M., Földi, A., Trábert, Zs., Ács, É., Darvas, B., Székács, A. (2017) Dissipation of the herbicide active ingredient glyphosate in natural water samples in the presence of biofilms. *Int. J. Envir. Anal. Chem.,* **97** (10): 901-921. (IF 1,208)

Székács, A. (2017) Environmental and ecological aspects in the overall assessment of bioeconomy. *J. Agric. Environ. Ethics,* **30** (1): 153-170 (impakt faktor: 1,162)

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Sági, Gy., Bezsenyi, A., Kovács, K., Klátyik, Sz., Darvas, B., Székács, A., Wojnárovits, L., Takács, E. (2018) The impact of H_2O_2 and the role of mineralization in biodegradation or

ecotoxicity assessment of advanced oxidation processes, *Radiat. Phys. Chem.*, **144**: 361-366. (IF: 1,315)

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Mörtl, M., Klátyik, Sz., Molnár, H., Tömösközi-Farkas, R., Adányi, N., Székács, A. (2018) The effect of intensive chemical plant protection on the quality of spice paprika. *J. Food Comp. Anal.*, **67**: 141-148. (IF 2,752)

Sági, Gy., Szabacsi, K., Szabó, L.,. Homlok, R., Kovács, K., Mohácsi-Farkas, Cs., Takács, E., Wojnárovits, L. (2018) Elimination of antibacterial activity of sulfamethoxazole and trimethoprim by ionizing radiation. *J. Environ. Sci. Health A*, accepted for publication. (IF:1,425)

Farkas, E., Szekacs, A., Kovacs, B., Olah, M., Horvath, R., Szekacs, I. (2018) Label-free optical biosensor for real-time monitoring the cytotoxicity of xenobiotics: a proof of principle study on glyphosate. *J. Hazard. Mater.*, under review (IF: 6,065)

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Székács, I., Fejes, Á., Klátyik, Sz., Takács, E., Patkó, D., Pomóthy, J., Mörtl, M., Horváth, R., Madarász, E., Darvas, B., Székács, A. (2014) Environmental and toxicological impacts of glyphosate with its formulating adjuvant. *Intl. J. Agric. Biosyst. Sci. Engineer.*, **8** (3): 1-6.

Székács, I., Horvath, R., Székács, A. (2016) Label-free optical biosensors for monitoring cellular processes and cytotoxic agents at interfaces using guided modes and advanced phase-contrast imaging techniques. In: *Biosensors for Security and Bioterrorism Applications* (Nikolelis, D. P., Nikoleli, G.-P., Eds.), Springer, Cham, Switzerland, pp. 443-468.

Klátyik, Sz., Bohus, P., Darvas, B., Székács, A. (2017) Authorization and toxicity of veterinary drugs and plant protection products: residues of the active ingredients in food and feed and toxicity problems related to adjuvants. *Front. Vet. Sci.*, **4**: 146. http://dx.doi.org/10.3389/fvets.2017.00146

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Szekacs, A., Komives, T. (2017) Research directions in plant protection chemistry. *Ecocycles*, 3 (2): 4-12.

Klátyik, Sz., Molnár, H., Pék, M., Bata-Vidács, I., Adányi, N., Székács, A. (2018) Quality management in spice paprika production: from cultivation to end product. In: Quality Management Systems (Kounis, L., Ed.), InTech. Rijeka, Croatia, accepted for publication.

Székács, A., Darvas, B. (2018) The re-registration of glyphosate in the European Union. *Front. Envir. Sci.*, accepted with major revision.

University and PhD dissertations prepared in the scope of the project:

Szabadi Nóra: *Glyphosate*-tartalmú készítmények és segédanyagaik víztoxikológiai vizsgálata (2013, BSc szakdolgozat). Szegedi Tudományegyetem, Természettudományi és Informatikai Kar

Fejes Ágnes: Perzisztens növényvédő szerek hatástartam- és lebomlásvizsgálatai (2015, PhD értekezés). Pannon Egyetem, Georgikon Kar, Állat- és Ágrárkörnyezet-tudományi Doktori Iskola

Füleki Lilla: *Glyphosate*-tartalmú gyomirtószerek és formázó anyagaik összehasonlító akut toxicitásvizsgálata vízi gerinctelen szervezeteken (2016, MSc Diplomamunka), Budapesti Műszaki és Gazdaságtudományi Egyetem, Vegyészmérnöki és Biomérnöki Kar

Mándics Ildikó: A *glyphosate*-hatóanyagtartalmú növényvédő szerek toxicitása vízi szervezeteken (2017, MSc Diplomamunka), Szent István Egyetem, Mezőgazdaság- és Környezettudományi Kar

Liang Xinyue: Ecotoxicological evaluation of organic micropollutants originated from agriculture and veterinary on great water flea (*Daphnia magna*) (2017, BSc thesis). Óbudai Egyetem, Rejtő Sándor Könnyűipari és Környezetmérnöki Kar

Jiang Dongze: Ecotoxicological evaluation of ROUNDUP CLASSIC herbicide formulation and its components on natural freshwater biofilms (2017, BSc thesis). Óbudai Egyetem, Rejtő Sándor Könnyűipari és Környezetmérnöki Kar

Huang Shikai: Analysis of surfactants used in agrochemicals (2017, BSc thesis). Óbudai Egyetem, Rejtő Sándor Könnyűipari és Környezetmérnöki Kar

Bolgár Evelin: (2018, MSc diplomamunka). Budapesti Műszaki és Gazdaságtudományi Egyetem, Vegyészmérnöki és Biomérnöki Kar

Klátyik Szandra: Mezőgazdasági kémiai és biológiai eredetű szennyezők toxikológiai vizsgálatai vízi szervezeteken (PhD értekezés). Szent István Egyetem, Mezőgazdaság- és Környezettudományi Kar, Biológia Tudományi Doktori Iskola, folyamatban (védés tervezett ideje: 2018)

Gyurcsó Gergő: Mezőgazdasági és városi mikroszennyezők vizsgálata zebradánió tesztállaton (PhD értekezés). Szent István Egyetem, Mezőgazdaság- és Környezettudományi Kar, Környezettudományi Doktori Iskola, folyamatban (védés tervezett ideje: 2020)

Oláh Marianna: Mezőgazdasági eredetű, endokrin zavaró és más toxikus hatász gyakorló vegyületek kimutatása és környezetanalitikai vizsgálatai (PhD értekezés). Szent István Egyetem, Mezőgazdaság- és Környezettudományi Kar, Környezettudományi Doktori Iskola, folyamatban (védés tervezett ideje: 2020)

Conference presentations and posters:

2014

Darvas, B., Bálint, A., Mörtl, M. Székács A. (2014): Pesticide residues in *Capsicum* species in the RASFF system (1998-2014). *Korean – Hungarian Workshop on Joint Research for Global Food Security: Ensuring Environmental and Food Safety* (Budapest), Abs. p.20.

Székács, I., Fejes, Á., Klátyik, S., Takács, E., Patkó, D., Pomóthy, J., Mörtl, M., Horváth, R., Madarász, E., Darvas, B., Székács, A. (2014): Environmental and toxicological impacts of glyphosate with its formulating adjuvant. *International Conference on Agricultural and Environmental Systems* (Miami, FL, USA), Abs. p.213.

Székács, I., Takács, E., Patkó, D., Horváth, R., Székács A. (2014): Holographic microscopy used in cellular toxicity assessment of agrochemicals. *Korean – Hungarian Workshop on Joint Research for Global Food Security: Ensuring Environmental and Food Safety* (Budapest), Abs. p.24.

Takács, E., Defarge, N., Séralini, G.-E., Székács A. (2014): Pesticide applications by and with genetically modified (GM) crops: misregulated pesticide issues. *Science in the Eye of the Storm - ENSSER Annual Conference 2014* (Berlin, Germany), Abs. 8.

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

2015

Klátyik, Sz., Földi, A., Ács, É., Cséffán, T., Pasaréti, Gy., Mörtl, M., Takács, E., Bohus, P., Székács, A., Darvas, B. (2015) Reaction of biofilms (Cyanobacterial, diatom and green algae) from lake Balaton and river

Danube to herbicide preparation ROUNDUP and its components (glyphosate, POEA). *Proc. 21st International Symposium on Analytical and Environmental Problems*, pp. 378-382.

Klátyik, Sz., Földi, A., Ács, É., Cséffán, T., Pasaréti, Gy., Mörtl, M., Takács, E., Bohus, P., Székács, A., Darvas, B. (2015) Balatoni és dunai biofilmek (kék-, zöld-, kovaalga) reakciója Roundup készítményre és összetevőire (*glyphosate*, POEA). *V. Ökotoxikológiai Konferencia* (Budapest, 2015. november 20.) pp. 17-19.

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Ottucsák, M., Klátyik, Sz., Gyurcsó, G., Mörtl, M., Darvas, B., Székács, A. (2015) Environmental monitoring of *glyphosate* and assessment of its combined cytotoxicity with adjuvants. *Proc. 21st International Symposium on Analytical and Environmental Problems* (Szeged, Hungary, September 28, 2015), pp. 64-67.

Ottucsák, M., Takács, E., Klátyik, Sz., Darvas, B., Székács, A. (2015) A környezet- és élelmiszer-biztonsági kockázatok súlyozása a *glyphosate* gyomirtó-hatóanyag és adjuvánsai példáján. *V. Ökotoxikológiai Konferencia* (Budapest, 2015. november 20.) pp. 28-29.

Székács, A., Mörtl, M., Darvas, B. (2015) Növényvédőszer-maradékok hazai felszíni vizekben: az elmúlt 25 év. *V. Ökotoxikológiai Konferencia* (Budapest, 2015. november 20.) pp. 38-39.

Székács, I., Patkó, D., Horváth, R., Székács, A. (2015) Holografikus mikroszkópia alkalmazása sejttoxicitás-vizsgálatokban. *45. Membrán-Transzport Konferencia* (Sümeg, 2015. május 19-22.), p. 107.

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Takács, E., Székács, I., Madarász, E., Darvas, B., Horváth, R., Székács, A. (2015) *Glyphosate* hatóanyagú növényvédő szer *in vitro* toxicitása emlőssejtvonalakon. *8. Magyar Sejtanalitikai Konferencia* (Budapest, 2015. szeptember 3-5.)

2016

Klátyik, Sz., Földi, A., Trábert, Zs., Pozderka, V., Ács, É., Cséffán, T., Pasaréti, Gy., Mörtl, M., Takács, E., Bohus, P., Székács A., Darvas B. (2016) A Roundup Classic gyomirtó szer és összetevőinek (*glyphosate*, POEA) hatása természetes biofilmek algaközösségeire. *VI. Ökotoxikológiai Konferencia* (Budapest, 2016. november 18.) pp. 17-18. poszter

Klátyik, Sz., Mörtl, M., Takács, E., Székács A., Darvas B. (2016) Neonikotinoid rovarirtószer-hatóanyagok és -készítmények hatása nagy vízibolhán (*Daphnia magna*). *VI. Ökotoxikológiai Konferencia* (Budapest, 2016. november 18.) pp. 19-20. poszter

Klátyik, Sz., Mörtl, M., Takács, E., Székács, A., Darvas, B. (2016) Effects of neonicotinoid insecticide formulations and their components on *Daphnia magna*. *9th European Conference on Pesticides and Related Organic Micropollutants in the Environment* and *15th Symposium on Chemistry and Fate of Modern Pesticides* (Santiago de Compostela, Spain, October 4-7, 2016) Book of Abstracts pp. 151-152. lecture OP40.

Klátyik, Sz., Székács A., Darvas B., Mörtl, M. (2016) A *glyphosate* gyomirtószer-hatóanyag lebomlása balatoni és dunai vízmintákban, valamint algabiofilm jelenlétében. *VI. Ökotoxikológiai Konferencia* (Budapest, 2016. november 18.) pp. 21-22. előadás

Klátyik, Sz., Trábert, Zs., Ács, É., Mörtl, M., Takács, E., Bohus, P., Székács, A., Darvas, B. (2016) The effects of herbicide preparation Roundup and its components (glyphosate, POEA) on the algal communities of natural biofilms. *9th European Conference on Pesticides and Related Organic Micropollutants in the Environment* and *15th Symposium on Chemistry and Fate of Modern Pesticides* (Santiago de Compostela, Spain, October 4-7, 2016) Book of Abstracts pp. 395-396. poster P121.

Mörtl, M., Darvas, B., Székács, A. (2016) Monitoring pesticide residues in Danube in Hungary: Surveys in 2011–2015. *18th DKMT Euroregion Conference* (Novi Sad, Serbia, June 2-4, 2016) p. 108.

Ottucsák, M., Kocsis, Zs., Bíró, A., Tolnai, Á., Darvas, B., Székács, A. (2016) A *comet-assay* alkalmazhatósága növényvédőszer-hatóanyag, -készítmény és formázó anyag toxicitásvizsgálatában. *VI. Ökotoxikológiai Konferencia* (Budapest, 2016. november 18.) pp. 27-28. poszter

Ottucsák, M., Mörtl, M., Székács, A. (2016) Comparative chemical analysis of environmental and cultivated paprika samples from ecological and conventional paprika crop fields. *SPICED Symposium* (Berlin, Germany, June 1-2, 2016) Abstracts p. 99.

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Székács, I., Klátyik, Sz., Darvas, B., Horváth, R., Székács, A. (2016) A *glyphosate* formázott készítményének és formázó anyagának citotoxicitási vizsgálatai holografikus mikroszkópia segítségével. *VI. Ökotoxikológiai Konferencia* (Budapest, 2016. november 18.) pp. 36-37. poszter

Székács, I., Klátyik, Sz., Darvas, B., Horváth, R., Székács, A. (2016) Cytotoxicological assessment of glyphosate, its herbicide product and the surfactant used for its formulation by holographic microscopy. *9th European Conference on Pesticides and Related Organic Micropollutants in the Environment* and *15th Symposium on Chemistry and Fate of Modern Pesticides* (Santiago de Compostela, Spain, October 4-7, 2016) Book of Abstracts pp. 393-394. poster P120

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Farkas, E., Székács, A., Kovács, B., Horváth, R., Székács, I. (2017) Study of the effects of xenobiotics on attached mammalian cell line by label-free biosensor. *International Conference on Nanomedicine and Nanobiotechnology – ICONAN 2017* (Barcelona, Spain, September 25-27, 2017), Oral presentation

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Gyurcsó, G., Simon, L., Bohus, P., Mörtl, M., Takács, E., Székács, A., Darvas, B., (2017) *Glyphosate* és formázó anyagainak hatása a *Danio rerio* embrionális fejlődésére. *VII. Ökotoxikológiai Konferencia* (Budapest, november 24.).

Klátyik, Sz., Földi, A., Ács, É., Takács, E., Mörtl, M., Székács, A., Darvas, B. (2017) A *glyphosate* gyomirtószer-hatóanyag hatásai a vízi életközösségekre. *Abs. III. Parlamenti Nyílt Napok a GMO-król* (Budapest, április 18-25.) pp. 65-66.

Klátyik, Sz., Földi, A., Ács, É., Takács, E., Mörtl, M., Székács, A., Darvas, B. (2017) A *glyphosate* gyomirtószer-hatóanyag hatásai a vízi életközösségekre. *VII. Ökotoxikológiai Konferencia* (Budapest, november 24.).

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Kotlán, B., Liszkay, G., Csuka, O., Tóth, J., Oláh, M., Kasler, M., Székács, A. (2017) Reduced tumor-associated glycosphingolipids in cell membrane lipid rafts and a diminished proliferation rate in mammary breast carcinoma cultures after incubation with glyphosate-based formulations, *Society for Immunotherapy of Cancer's (SITC) 32nd Annual Meeting* (National Harbour, MD, USA, November 8-12.)

Mörtl, M., Székács, A. (2017) Felületaktív anyagok analízise környezeti mintákban. *6. Környezetkémiai Szimpózium* (Bakonybél, október 12-13.) előadás, p. 12.

Mörtl, M., Székács, A. (2017) Determination of surfactants used in agrochemicals. *Proc. 23rd International Symposium on Analytical and Environmental Problems* (Szeged, Hungary, October 9-10, 2017) pp. 62-66.

Mörtl, M., . Takács, E ., Klátyik, Sz., Székács A. (2017) Determination of surfactants used in agrochemicals. *18th European Meeting on Environmental Chemistry (EMEC)* (Porto, Portugal, November 26-29, 2017)

Mörtl M., Takács E., Klátyik Sz., Székács A. (2017) Növényvédő szerek tapadásfokozó adalékanyagainak meghatározása. *XIII. Környezetvédelmi Analitikai és Technológiai Konferencia* (Debrecen, 2017. augusztus 23-25) KAT12-P02

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Oláh, M., Takács, E., Kocsis, Zs., Darvas. B., Székács, A. (2017): A *glyphosate* és készítményeinek hatása sejtvonalakon. *VII. Ökotoxikológiai Konferencia* (Budapest, november 24.).

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