HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

ECOTOXICOLOGICAL AND BIODEGRADATION ASSESSMENT OF COMMON ENVIRONMENTAL HERBICIDES

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1. SCIENTIFIC BACKGROUND AND OBJECTIVES

In order to protect our crops and animals, increase yields and improve the quality of products, mankind currently uses 3 million tons of pesticides globally every year. Their use has been increasing gradually since the mid-20th century. In 2020, according to the latest report on pesticide use in Hungary, 28.9 thousand tons of pesticides were placed on the market, of which 40.6% were herbicides, 28.3% were fungicides, 14.4% were soil disinfectants and 9.5% were insecticides. However, as a consequence of intensive use, residues have been detected in virtually every environmental compartment -except for food raw materials and finished products-from the Arctic to Antarctica, and the long-term human and environmental health effects are often unknown. Through their secondary effects (persistence, acute and chronic toxicity to non-target organisms), they pose a risk to living organisms exposed to them. They also contribute to the process of environmental damage and pollution.

Advanced methods for assessing their adverse biological/toxicological/ecotoxicological effects have been available since the 1990s and can be used to detect not only acute but also chronic toxic effects, even when xenobiotics are present in the environment at relatively low concentrations (ng/L-µg/L). In the longer term, interactions between pesticides and other pollutants or compounds may lead to antagonistic, synergistic, or additive processes. However, our understanding of these interactions is still very incomplete.

In order to enhance the broad-spectrum herbicidal effect; the active substances are "potentiated" with members of a herbicide group with different biological mechanisms of action. A typical example of this is the active substance mesotrione coated with terbuthylazine and S-metolachlor. In addition, glyphosate, the most widely used and marketed herbicidal active substance in the world, and glyphosate-based formulations are also the subject of much attention because of

their toxicological concerns. The problem is thought to be caused by co-formulants and surfactants in the formulations.

Pesticides are typically released into the environment by their agricultural, horticultural/and gardening applications. Siltation of run-off from fields, over-irrigation, extreme high rainfall (sudden, rapid rainfall) and surface run-off caused by climate change contribute to the increasing leaching of chemicals used in agriculture into deeper soil layers and groundwater bodies. A significant proportion of these chemicals also pollute natural water bodies. This can be a problem, as pesticide active substances that are degraded in the upper, ploughed soil layer over a relatively short period, can accumulate in the groundwater and sediments in the absence of UV light and oxygen in a so-called secondary persistence process, unchanged for decades, retaining their harmful/toxic chemical and biological activity. A further problem may be that in many cases their degradation under unfavourable conditions may result in the formation of degradation products or residues that are significantly more toxic than the parent compound. From a cost-effective point of view, bioremediation techniques are appropriate for the elimination of these compounds through biodegradation studies. Biologically based ecotoxicological tests are the best option for monitoring the metabolites formed from the parent compound from the perspective of impact assessment (biodetoxification).

To determine the ecotoxicity, biodegradability and biodetoxification of these active substances of pesticides and their mixtures, as well as of some formulations, I have defined the following objectives in my doctoral thesis:

I. Active substances in different combinations in herbicide formulations which are among the most widely used in the European Union:
- mesotrione, S-metolachlor, terbuthylazine and their mixtures in various proportions,

- the active substance glyphosate and its main degradation product AMPA, and glyphosate-based formulations,
- POE(15) co-formulant

assessment of cell toxicity by acute and chronic exposure using the *Aliivibrio fischeri* ecotoxicological test for acute and chronic cytotoxicity.

II. Exploration of potential direct hormone effects of mesotrione, S-metolachlor, terbuthylazine and mixtures of these active substances, as well as glyphosate, AMPA, POE(15) co-formulant and glyphosate-based formulations [with and without POE(15)] with *Saccharomyces cerevisiae*-based bioreporters.

III. For combined mixtures of active substances, the Combination Index Method is used to determine synergistic, additive, and antagonistic effects on the test organism used.

IV. Isolation and identification of bacterial strains that potentially biodegrade glyphosate, mesotrione, S-metolachlor, and terbuthylazine from contaminated environmental compartments and the continuous expansion of the existing strain collection at the Department of Environmental Safety (DES).

V. *In vitro* investigation of the biodegradation and biodetoxifying capacity of bacterial strains isolated and identified from contaminated environmental compartments and of *Rhodococcus* strains with pesticide active substance degrading properties as described in the scientific literature, in aerobic and oxygen-limited environments.

VI. Comparison of the biodegradation dynamics of glyphosate, mesotrione, S-metolachlor and terbuthylazine under oxygen-saturated and oxygen-limited conditions.

VII. Investigation of the adverse biological/ecotoxicological effects of degradation residues produced by bacterial strains with good degradation potential from herbicide active substances under aerated and oxygen-limited conditions.

2. MATERIALS AND METHODS

Materials used

From an ecotoxicological and biodegradation point of view, I have investigated the pure compounds mesotrione, S-metolachlor, terbuthylazine, glyphosate, AMPA and POE(15). For my investigations, I prepared eight mixtures of mesotrione, S-metolachlor and terbuthylazine at 1:1 and 1:1:1 ratios of active ingredients and herbicide formulations at active ingredient ratios. I also investigated 13 glyphosate-based formulations that are freely available and obtainable by anyone in the Hungarian pesticide trade.

Biological impact assays

Their short- and long-term cytotoxic effects were determined using the standard (ISO 11348-2) acute Microtox® *Aliivibrio fischeri* (*AVF*) bioluminescence inhibition assay and the chronic version of the modified, prolonged contact time microtiter plate adapted assay. Androgenic and estrogenic effects were determined using genetically modified *Saccharomyces cerevisiae* BLYAS, BLYES yeast test organism strains. In the cases of BLYAS and BLYES test organisms, the intensity of bioluminescence are directly proportional to the degree of hormonal activity of the compound. For measurement of cytotoxicity, I used the constitutive BLYR strain, whose light emission decreases in response to toxicity, in parallel with the BLYAS/BLYES strains.

Statistical analysis, evaluation

Data from the biological impact assays were statistically analysed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). Determination of concentration-response curves and calculation of EC_x values were performed using the software. Concentration-response curves were generated by non-linear regression fitting, obtained after logarithmic transformation and % normalization of the data. When calculating the

bioluminescence inhibition and intensification values, I calculated the percentage decrease or increase of the samples' light emission values from the control.

Combination Index Method

Antagonism, addition, and synergism between compounds were determined using the Combination Index Method. The Combination Index (CI) values quantifying the synergistic, additive, and antagonistic effects of mixtures of active compounds were quantified using CompuSyn software (The ComboSyn, Inc.). The effects of the mixtures were classified into a detailed CI range system based on the obtained CI values and classified qualitatively according to the strength of the combined effects.

Sampling locations and sampling

Between 2013 and 2019, environmental samples (surface- and subsurface water, soil, sediment) were collected to isolate bacterial strains that can potentially biodegrade and biodetoxify herbicide active substances from i.) a hydrocarbon-contaminated site in Central Hungary, ii.) from an agricultural area in Cegléd, and iii.) from inflow- and outflow watercourses in the Balaton sub-catchment area.

Isolation and identification of microbes

From the environmental samples collected, I created enrichment media (artificially contaminated with herbicides) from which microbes isolated that could potentially degrade herbicidal active ingredients using general microbial culture procedures. The isolates were identified by 16S rDNA gene sequence analysis using molecular biology methods.

Biodegradation (aerobic and microaerobic) and biodetoxification experiments

In the biodegradation experiments used, I selected the microbes isolated and identified, and those that my literature research has shown to be capable of biodegrading xenobiotics, considering their hazard classification from a human- and environmental health perspectives. From the strains, a 72-hours inoculum of

cell density (OD_{600}=0.6) was generated, and the degradation of herbicide active substances at 5 mg/L was investigated in liquid submerged cultures. At the end of the degradation experiment (7 days incubation with shaking at 28 °C), bacterial suspensions were separated into supernatant and pellet fractions by centrifugation for subsequent chemical-analytical (GC-MS/MS and/or LC-MS/MS, Wessling Hungary Ltd.) and biodetoxification (chronic *AVF*) experiments. The biodegradation or bioadsorption capacity of the strains was expressed as a percentage compared to the control samples. In my degradation experiments under microaerobic/oxygen-limited conditions, I used bacterial strains that had at least 40% degradation potential in my experiments under aerobic/oxygen-limited conditions based on results from three replicates in parallel. The cytotoxicity (biodetoxification) of the degradation residues was measured using the chronic *AVF* test.

3. RESULTS

Acute and chronic Aliivibrio fischeri cytotoxicity tests (mesotrione, S-metolachlor, terbuthylazine)

The acute toxicity of mesotrione, S-metolachlor, and terbuthylazine active substances is in ascending order: terbuthylazine (non-toxic), S-metolachlor (265 mg/L), mesotrione (118 mg/L). Mixtures mixed in the same ratio (1:1) resulted in higher toxicity than those mixed according to the formulations. Of the 1:1 mixtures, the non-toxic terbuthylazine combined with mesotrione produced the highest toxicity (EC_{50}= 30 mg/L). Furthermore, it was found to be more than ten times more toxic than when combined with S-metolachlor (EC_{50}= 376 mg/L) (**Table 1**).

In the chronic *AVF* test, the test organism was found to be most sensitive and reliable at exposure times of 10 and 15 hours. The chronic toxicity of S-metolachlor was an order of magnitude greater ($EC_{50,\ 10h}$= 59.2 mg/L, $EC_{50,\ 15h}$= 54.0 mg/L) than that measured in the acute test ($EC_{50,\ 30min}$= 265 mg/L), while there was no significant difference in the toxicity of mesotrione ($EC_{50,\ 10h}$= 75.9 mg/L, $EC_{50,\ 15h}$= 189.2 mg/L) compared to the results of the acute test ($EC_{50,\ 30min}$= 118 mg/L). Similar to S-metolachlor, *AVF* showed a notably increased sensitivity to terbuthylazine ($EC_{50,\ 10h}$= 4.9 mg/L and $EC_{50,\ 15h}$= 9.6 mg/L). In the chronic *AVF* test, terbuthylazine showed the strongest toxic effect on the test organism. Three of the formulation-based mixtures (Gardoprim Plus Gold®, Calaris Pro® and Lumax®) were found to be an order of magnitude more potent in the chronic test than in the short-term Microtox® test. In contrast to the acute test, no significant difference in toxicity of the same magnitude was found between the mixtures in the proportions of active substances indicated in the formulations and those mixed at the same ratios in the chronic test (**Table 1**).

Table 1: EC_{50} values (mg/L) of the acute (30 minutes) and chronic (10 and 15 hours) *AVF* tests compared to the literature with acute *AVF* tests.

Active ingredients of pesticides and their mixtures	EC_{50} values (mg/L) from chronic *AVF* assay (95% confidence intervals) and R^2 values in our study		EC_{50} values (mg/L) from acute *AVF* assay (95% confidence intervals)	
	10 hours	15 hours	In our study	Present in literature
mesotrione	75,9 (64.21 – 89.64) R^2: 0.917	189.2 (161.7 – 221.3) R^2: 0.906	118 (76 – 180)	43.6 ± 2.4 (15 minutes); 398.66
S-metolachlor (*metolachlor)	59.2 (52.36 – 69.02) R^2: 0.924	54.0 (49.08 – 61.50) R^2: 0.935	265 (190 – 370)	17 (**metolachlor*); 214.5; 178.4 ± 22.8 (15 perc); 215.3
terbuthylazine	4.9 (4.03 – 6.10) R^2: 0.920	9.6 (6.57 – 14.07) R^2: 0.679	n.t. (-)	41.07 (15 minutes); n.d.
S-metolachlor + terbuthylazine (ratio 1:1)	117.2 (83.78 – 163.9) R^2: 0.843	111.7 (87.34 – 142.8) R^2: 0.835	376* (168 – 838)	n.d.
S-metolachlor + terbuthylazine (ratio[1] 1.7:1)	48.1 (34.25 – 67.68) R^2: 0.851	52.4 (33.46 – 82.20) R^2: 0.740	450* (84 – 2380)	n.d.
mesotrione + S-metolachlor (ratio 1:1)	100.7 (63.85 – 158.8) R^2: 0.870	87.2 (57.53 – 132.3) R^2: 0.868	50 (38 – 66)	14.4 ± 0.8 (15 minutes)
mesotrione + S-metolachlor (ratio[2] 1:8.5)	112.3 (68.85 – 183.2) R^2: 0.880	102.9 (46.44 – 227.9) R^2: 0.717	182 (86 – 386)	n.d.
mesotrione + terbuthylazine (ratio 1:1)	34.2 (22.40 – 52.33) R^2: 0.686	38.5 (28.01 – 53.01) R^2: 0.780	30 (24 – 34)	n.d.
mesotrione + terbuthylazine (ratio[3] 1:6.5)	16.4 (12.55 – 21.38) R^2: 0,738	19.5 (13.44 – 28.16) R^2: 0.612	212 (86 – 512)	n.d.
mesotrione + S-metolachlor + terbuthylazine (ratio 1:1:1)	55.5 (34.60 – 89.23) R^2: 0.819	59.5 (41.12 – 84.12) R^2: 0.790	56 (46 – 68)	n.d.
mesotrione + S-metolachlor + terbuthylazine (ratio[4] 1:10:3)	79.8 (54.78 – 116.4) R^2: 0.920	81.0 (51.82 – 126.8) R^2: 0.837	136 (44 – 410)	n.d.

Legend: [1]: based on the content of Gardoprim Plus Gold® (Syngenta AG - 312 g/L S-metolachlor, 187 g/L terbuthylazine); [2]: based on the content of Camix® (Syngenta AG – 60 g/L mesotrione, 500 g/L S-metolachlor); [3]: based on the content of Calaris Pro® (Syngenta AG – 50 g/L mesotrione, 326 g/L terbuthylazine); [4]: based on the content of Lumax® (Syngenta AG – 37.5 g/L mesotrione, 375 g/L S-metolachlor, 125 g/L terbuthylazine); n.t.: not toxic - toxic effect (inhibition in bioluminescence) was not found at the tested concentrations n.d.: no data was found; n = 5 in the acute test, n = 3 in the chronic test; *: EC_{50} value was calculated from extrapolated data.

Combined effects of active substance mixtures determined by the Combination Index Method

Acute *AVF*: After 30 minutes of exposure, all mixtures showed synergistic effects with CI values (EC_{50}) ranging from 0.12 to 0.77, indicating strong to moderate synergism. The strong synergism of mixtures containing terbuthylazine is probably due to its non-toxic effects in itself. However, combined acute toxicity (EC_{50}= 30 mg/L) of mesotrione and terbuthylazine and their synergism at the ratio of 1:1 is the greatest among all the examined chemicals and mixtures. Among the CI values determined at EC_{90}, two combinations of S-metolachlor + terbuthylazine (1:1 and 1:1.7) and mesotrione + terbuthylazine (1:6.5) showed strong and moderate antagonistic effects, respectively. Mesotrione mixed with S-metolachlor at the same proportion demonstrated strong synergism with a notable CI value (0.12) even at low concentrations (EC_{10} and EC_{20}); whereas the ratio of 1:8.5 showed an antagonistic and additive response.

Chronic *AVF*: the mixture of mesotrione + S-metolachlor (1:1) indicated additive/synergistic effects. The Combination Index Method revealed antagonistic responses caused by all the other mixtures and combinations at both contact times (10 and 15 hours) with moderate (CI >1.20) to very strong (>10) antagonism.

Acute and chronic Aliivibrio fischeri cytotoxicity tests [glyphosate, AMPA, POE(15), glyphosate-based formulations (13)]

After 30 min, glyphosate and AMPA as pure chemicals did not cause bioluminescence inhibition to *AVF* even at the highest applied concentrations. Moreover, a slight elevation in the bioluminescence could be observed at the lowest concentrations in the case of AMPA and POE(15). Glialka Express 6H® exerted the most toxic effects, where the EC_{50} values were 3.8 and 3 mg/L for the glyphosate IPA-salt and glyphosate-acid, respectively. Glialka Express 6H® was followed by Roundup® Classic, Total®, Roundup® Mega, Medallon Premium® and Glialka Star®. The toxicity of Roundup® Mega and Glialka Star® was very

similar due to their almost identical composition. The other glyphosate-based herbicides were less toxic to *Aliivibrio fischeri* in the acute test: the EC_{50} values were by one order of magnitude higher, from 144 (108 mg/L for glyphosate-acid) to 606 (445 mg/L for glyphosate-acid) mg/L.

Regarding the chronic assay, the toxicity of POE(15) was similar to the one in the acute assay. Glyphosate and AMPA as pure substances also did not cause any inhibition, same as in the acute test. Glialka Express 6H® also proved to be the most toxic glyphosate-based herbicide, with EC_{50} values at 3.9 and 1.2 mg/L after 10 and 15 hours of exposure, respectively. It is followed by Barclay Gallup Biograde 360® and Medallon Premium®, where the EC_{50} values were also very low: 4.5 and 39.3 mg/L after 10 hours and 26.4 and 30.3 mg/L after 15 hours, respectively. *AVF* showed the highest sensitivity after 15 hours of herbicide exposure in the chronic assay and was more sensitive toward pesticides than in the acute test.

Overall, a correlation could not be found between cytotoxicity and the type of glyphosate salt in the formulations. Therefore, the high toxicity is most likely caused by the co-formulants and not by the glyphosate salt.

Hormonal activity and cytotoxicity assays with Saccharomyces cerevisiae-based bioreporters (BLYAS, BLYES, BLYR)

None of the active substances mesotrione, S-metolachlor, and terbuthylazine induced light emission in BLYAS and BLYES bioreporters. Thus, it can be concluded that none of the active substances has a direct hormonal activity, i.e., they do not bind directly to human oestrogen or androgen receptors.

Similarly, to the cytotoxicity results, neither glyphosate nor AMPA proved to be toxic to BLYR or hormonally active in the case of BLYES and BLYAS strains. POE(15) had cytotoxic effect (EC_{50}= 24 mg/L) and estrogenic activity (EC_{50}= 13.4 mg/L) on BLYR and BLYES, respectively. Glialka Express 6H® as in the *AVF* assays had a strong toxic effect on the *S. cerevisiae* BLYR strain with a very low EC_{50} value (4.5 mg/L). Due to the cytotoxicity, the hormonal activity could

not be measured by BLYES or BLYAS. POE(15) containing Gladiator 480 SL® and Roundup® Classic alongside Total® and Glialka Star® formulated with other co-formulants had moderate toxicity and all of them resulted in bioluminescence intensification in BLYES strain. Glyfos Dakar® was the only glyphosate-based herbicide which had neither cytotoxic effects nor estrogenicity but exhibited androgenic activity (EC50= 22.4 mg/L). Fozat 480®, Dominator Extra 608 SL® and Roundup® Mega proved to be slightly toxic and had not only estrogenic but androgenic activity as well. Regarding the results from the bioreporter assays, estrogenic and/ or androgenic effects cannot be attributed to the type of glyphosate salt.

Cytotoxicity and hormonal activity of glyphosate-based herbicides expressed as a percentage of dilution compared to recommended values for agricultural and household use

Dilution percentages (%) converted from *Aliivibrio fischeri* and *Saccharomyces cerevisiae* BLYR cytotoxicity and BLYES and BLYAS hormonal activity effective concentration values (mg/L) calculated from the glyphosate-salt content of glyphosate-based formulations are shown in **Table 2**. Almost all the formulations had a cytotoxic effect on *Aliivibrio fischeri* at a dilution of one or two orders of magnitude smaller than the minimum recommended value for agricultural and/or household (0.2 – 3.5%) use. A similar phenomenon could be observed when measuring direct hormonal activity: while Medallon Premium® had estrogenic activity at a relatively high (16%) dilution, the other glyphosate-based herbicides have hormonal activity at a dilution of 0.011 - 0.41%. The EC_{50} value for the androgenic activity of Roundup® Mega occurred at a dilution of three orders of magnitude lower (7.15E-04% of the original formulation) than the minimum recommended dilution (0.2 – 3.5%) for agricultural use.

Table 2: EC_{50} values for cytotoxicity and hormonal activity converted to dilution percent for glyphosate-based herbicides.

Formulations	Calculated level of dilution (%) for formulations regarding EC_{50} values					
	Acute *AVF* 30 minutes	Chronic *AVF*		BLYES strain 5 hours	BLYAS strain 5 hours	BLYR strain 5 hours
		10 hours	15 hours			
Barclay Gallup Biograde 360® [1]	0.125	0.0009	0.0055	n.e.	n.e.	n.e.
Boom Efekt® [1]	0.125	0.103	0.061	n.e.	n.e.	1.59
Fozat 480® [1]	0.03	0.065	0.017	0.035	0.011	0.23
Gladiator 480 SL® [1, 6]	0.03	0.078	0.021	0.018	n.e.	0.10
Glialka Express 6H® [1]	0.04	0.041	0.012	n.m.	n.m.	0.046
Kapazin® [1]	0.06	0.061	0.026	n.e.	n.e.	n.e.
Roundup® Classic [1, 6]	0.01	0.080	0.024	0.011	n.e.	0.021
Total® [1]	0.015	0.009	0.040	0.015	n.e.	0.021
Glialka Star® [3]	0.02	0.141	0.042	0.029	n.e.	0.029
Roundup® Mega [3]	0.015	0.079	0.025	0.023	7.15E-04	0.025
Dominator Extra 608 SL® [2]	0.026	0.056	0.029	0.041	0.012	0.29
Glyfos Dakar® [4]	0.04	0.011	0.083	n.e.	0.003	8.42
Medallon Premium® [5]	0.02	0.008	0.006	16.13	n.e.	n.e.

Legend: [1] – IPA-salt; [2] – DMA-salt; [3] – P-salt; [4] – AM-salt; [5] – DIAM-salt; [6] – POE(15) containing formulation; n.e. – no effect; n.m. – not measurable because of cytotoxic effect.

Biodegradation and biodetoxification experiments

In my biodegradation experiments, I included forty-five strains of thirty-six microbial species belonging to twenty different genera, based on my scientific literature research. In addition, forty-four type strains belonging to the genus *Rhodococcus* from international collections were also investigated.

In biodegradation experiments under aerobic conditions, none of the ninety-nine bacterial strains tested could biodegrade glyphosate, mesotrione and terbuthylazine. *Streptomyces caniferus* strain K176 degraded S-metolachlor with excellent efficiency (94.6% ± 1.1%). In the case of K176, the degradation was confirmed by the fact that the initial concentration of S-metolachlor adsorbed on the pellet was less than 1% of the initial concentration. *Streptomyces caniferus* was able to biodetoxifying the compound by detecting an increase in light emission, i.e., cytotoxicity cessation, at 10 and 15 hours in the chronic *AVF* test. Between ~40 and 65% of the initial concentration of terbuthylazine (5 mg/L) was bioadsorbed on the biomass of three *Rhodococcus* strains (*R. kyotonensis* JCM 23211, *R. phenolicus* JCM 14914, *R. agglutinans* KCTC 39118). Due to the bioadsorption processes, the amount of residual terbuthylazine remaining in the supernatant (~2-3 mg/L) was not cytotoxic in the chronic *AVF* bioassay, generating a light emission intensification.

Under microaerobic conditions, none of the bacterial strains was able to biodegrade any of the four active substances. As a consequence of the unsuccessful microaerobic biodegradation experiments, the biodetoxification capacity of the microbes was not tested.

4. CONCLUSIONS AND SUGGESTIONS

In my dissertation, I focused on the ecotoxicity of active substances of herbicides, mixtures, and formulations, and the biodegradability and biodetoxification of active substances. Among the herbicides, I selected glyphosate, mesotrione, S-metolachlor and terbuthylazine, which are frequently detected in the environment.

To evaluate the cytotoxicity of herbicides, I used the standard acute *AVF* test. I found that terbuthylazine did not, but S-metolachlor and mesotrione did produce acute toxicity. Several experimental studies have previously investigated the microbial ecotoxicity of these herbicides in the acute Microtox® test or a mixture of these (mesotrione + S-metolachlor), comparing them with my results, good agreement was observed in the S-metolachlor. However, for terbuthylazine, I obtained contradictory results to the literature data (terbuthylazine: $EC_{50,\ literature}$= 41.07 mg/L (15 minutes), $EC_{50,\ own\ measurement}$= non-toxic). The large differences observed could be due to the fact that the authors exposed the test organism for 15 minutes instead of 30 minutes. Mesotrione and S-metolachlor caused bioluminescence inhibition in both acute and chronic tests, although the toxic effect was more pronounced in the latter, similar to terbuthylazine. The short- and long-term toxicity of combinations of mesotrione, S-metolachlor and terbuthylazine [Microtox® test, except mesotrione + S-metolachlor (1:1)] was demonstrated for the first time. Of the eight mixtures, one mixture with the same active ingredient content as an authorised formulation currently available on the pesticide market (Calaris Pro®) was the most toxic (mesotrione + terbuthylazine 1:6.5). As regards the chronic cytotoxicity of the active substances, the microtiter plate method for *AVF* is more time-efficient than the acute test and allows a larger number of samples to be evaluated simultaneously per unit time. Based on the results, the sensitivity of the chronic prolonged-time method was found to be better (by up to one order of magnitude) than the 30-minute standard acute test.

The chronic bioassay using the *AVF* prokaryotic test organism is a sensitive and easy-to-perform test for the determination of long-term chronic toxicity of pesticides. The increased sensitivity of the chronic test is an important result for a more accurate assessment of the ecological risk of pesticides and their mixtures. The above-mentioned advantages justify the routine use of the chronic *AVF* test in practice, and it could be easily established as a component of ecotoxicological studies. In the future, it could be useful as a preliminary and stand-alone warning and assessment test method.

The largest database for joint effects of pesticides was created by BELDEN et al. (2007) providing data on 207 pesticide mixtures: mesotrione, S-metolachlor, and terbuthylazine could be found in the database; however, there are no data on mixtures of these herbicides. This is also the first report on the joint effects of mixtures of these herbicides, which had an antagonistic, additive, or synergistic effect on the *AVF* test organism.

The adverse biological effects of glyphosate-based herbicides have been reported in many studies, yet controversy remains. I focused my studies on eleven free-marketed POE(15)-free formulations which exhibited acute and chronic cytotoxicity (AVF tests) and direct estrogenic and androgenic effects (BLYES and BLYAS tests), while the pure active ingredient glyphosate acid proved to be ineffective in the applied biotests. I report the first *in vitro* data of acute and chronic cytotoxicity of glyphosate-based formulations without POE(15) on the ecotoxicological test organism *Aliivibrio fischeri* and their direct estrogenic and androgenic effects with *Saccharomyces cerevisiae* yeast-based bioreporters. No relationship was found between biological effects and glyphosate salt type or concentration. It can be concluded that cytotoxicity and endocrine activity may be related to the co-formulants present in the formulations. According to my findings, all the thirteen investigated GBHs were cytotoxic to *Aliivibrio fischeri* in both acute and chronic tests. Ten of them possessed direct hormonal activity at a dilution of at least one order of magnitude lower than the recommended

dilutions in the directions for agricultural and household use or at concentrations 0.5% that was found in surface and groundwaters after their application. The most pronounced hormonally active effects were observed below the 0.5% concentration level, in several cases at levels one order of magnitude lower. Based on my results, I compared the EC_{50} values from chronic *AVF* tests with those for other aquatic organisms as specified in their material safety data sheets. Remarkable differences were found for *Daphnia magna*: the EC_{50} values measured by *AVF* were one and two orders of magnitude lower than those measured by *Daphnia magna* 48-hour assay in the case of eight formulations. The results show that in addition to the active substance, a reassessment of formulations containing the active substance cannot be missed. In addition to the ecotoxicological study of pesticide active substances and formulations, the revision of the toxicity of co-formulants is also essential. It is essential to understand the adverse and cumulative effects on health and environment of the co-formulants applied in glyphosate-based formulations for a more comprehensive risk assessment, update their evaluation protocols (potential carcinogenicity, endocrine disruption effects) and revise the free availability to any one of these products, which is also an urgent task.

Of the ninety-nine bacterial strains investigated in aerobic biodegradation, only one was able to almost completely degrade S-metolachlor, suggesting that even under favourable and controlled laboratory conditions, the compounds are difficult to biodegrade. The chemical-analytically confirmed biodegradation was followed by the measurement of the cytotoxicity of the degradation residues produced by the microorganisms: the strain that successfully degraded S-metolachlor biodetoxified it.

The binding of terbuthylazine on *Rhodococcus* pellets is an interesting new scientific finding. Reviewing the literature, I found no reference to the phenomenon of terbuthylazine binding on the cell wall of members of the genus. Monitoring the binding of these molecules on microbial cells is a key task for

future *in vitro* studies. I hypothesized that the biodegradation activity of *Rhodococcus* was strongly influenced by the terbuthylazine binding phenomenon on bacterial pellets.

Biodegradation under oxygen-limited conditions did not lead to positive results. S-metolachlor was found to be persistent and difficult to degrade in the presence of low oxygen concentrations (≤ 0.5 mg/L). The microbes that successfully degraded the compound aerobically were no longer able to degrade it under microaerobic conditions, indicating a phenomenon of secondary persistence. In the future, it may also be useful to develop microaerobic/anaerobic isolation and enrichment techniques to identify microbes that have the potential to biodegrade and thus mitigate secondary persistence. The presence and abundance of genes encoding enzymes that catalyse the degradation of contaminants may be the most direct predictor of the biodegradation capacity of microbes. Modern molecular biology tools can be used to investigate individual chromosomal or extra-chromosomal (plasmid-mediated) functional genes. Genome sequencing and gene expression experiments can provide an excellent basis for accurate mapping and understanding of degradation capabilities.

In addition to the study of pesticide-active substance cocktails, further attention should be paid to the overall biological and toxicological effects of co-formulants and excipients, as well as pesticide formulations (including those in the category I and II marketed under authorisation) and their biodetoxification potential.

5. NEW SCIENTIFIC RESULTS

Thesis I.: Effective concentration values for the acute (30 minutes) and chronic (10 and 15 hours) *Aliivibrio fischeri* test organism were first determined for different combinations of mesotrione, S-metolachlor and terbuthylazine herbicide active substances in different ratios (except for the 1:1 mixture of mesotrione + S-metolachlor in the acute Microtox® test). The *Aliivibrio fischeri* test organism was found to be most sensitive in the chronic test at 10 and 15 hours contact times, with terbuthylazine ($EC_{50\ 10\ and\ 15\ hours}$ = 4.9 and 9.6 mg/L) producing the highest toxicity of the pure active substances and the Calaris Pro® formulation of mesotrione + terbuthylazine ($EC_{50\ 10\ and\ 15\ hours}$ = 16.4 and 19.5 mg/L) producing the highest toxicity of the mixtures. The chronic EC_{50} of the three formulation mixtures containing mesotrione and S-metolachlor beside terbuthylazine was an order of magnitude lower than the acute test. The chronic test using the prokaryotic test organism is a suitable stand-alone control/evaluation test method for the assessment of mixture effects.

Thesis II.: The Combination Index Method was used to determine for the first time the antagonistic, additive, and synergistic effects between the compounds of different proportions of mixtures of mesotrione, S-metolachlor and terbuthylazine at different effective concentration levels in acute and chronic *Aliivibrio fischeri*. In the acute test, synergism was detected at 50% toxic concentration (EC_{50}) for all mixtures. The mixture of mesotrione and S-metolachlor in the same ratio (1:1) showed additive and synergistic effects at all effective concentrations (even at EC_{10} = 5.1 mg/L) in the acute test, and at EC_{50}, EC_{80}, EC_{90} in the chronic test (at 10 and 15 hours contact time). In the chronic test, the other mixtures produced an antagonistic response at all effective concentrations.

Thesis III.: For glyphosate, AMPA, POE(15) pure compounds and thirteen glyphosate-based herbicide formulations, I described for the first time the chronic

cytotoxic effects of *Aliivibrio fischeri* on the ecotoxicological test organism and the acute cytotoxic effects of twelve formulations (excluding Roundup® Classic). In the acute and chronic tests, glyphosate acid and the metabolite AMPA were not toxic at the highest concentration used (5 g/L). In contrast, eleven formulations at concentrations between 44 and 623 mg/L showed 50% toxicity in the short- and long-term tests. In terms of glyphosate salt content, the ready-to-use Glialka Express 6H® ($EC_{50\ 10\ and\ 15\ hours}$ = 3.9 mg/L and 1.2 mg/L) and Barclay Gallup Biograde 360® ($EC_{50\ 10\ and\ 15\ hours}$ = 4.5 mg/L and 26.4 mg/L) with undeclared co-formulants and excipients were found to be the two most toxic glyphosate-based formulations. No correlation was found between cytotoxic effects and the glyphosate salt types in the formulations.

Thesis IV.: Using the *Saccharomyces cerevisiae* strain BLYES, I demonstrated that the co-formulant POE(15) has estrogenic activity (EC_{50}= 13.4 mg/L) and therefore, it is able to bind to the human oestrogen receptor, whereas glyphosate and AMPA are not. I first reported *in vitro* results on the direct estrogenic and androgenic effects of the tested formulations as measured by the *Saccharomyces cerevisiae* BLYES and BLYAS tests. Of these products, seven were found to be estrogenic, four androgenic and three (Fozat 480®, Dominator Extra 608 SL®, Roundup® Mega) were found to be estrogenic and androgenic.

Thesis V.: Based on the EC_{50} values of the acute and chronic *Aliivibrio fischeri* tests and the *Saccharomyces cerevisiae* BLYES/BLYAS bioreporter tests, thirteen and ten preparations have cytotoxic and endocrine-disrupting effects at concentrations of one or two orders of magnitude (0.01-0.1%) lower than the dilution rate used under agricultural and household conditions (0.2-3.5%) and at concentrations below the levels detectable in the environment (0.5%). In addition to the evaluation of active substances, I have demonstrated that a complex toxicological/ecotoxicological review of free-marketed and commercially available formulations is urgently needed to identify their human and

environmental health risks, as co-formulants/excipients influence the toxicological effects.

Thesis VI.: In biodegradation experiments under aerobic conditions, I demonstrated for the first time that *Streptomyces caniferus* strain K176 can degrade S-metolachlor by almost 95% without producing residues that are toxic to chronic *Aliivibrio fischeri* test. However, strain K176 is unable to biodegrade and biodetoxify the compound under microaerobic conditions. In the case of terbuthylazine, I demonstrated for the first time that *Rhodococcus kyotonensis* JCM 23211^{T}, *Rhodococcus phenolicus* JCM 14914^{T}, *Rhodococcus agglutinans* KCTC 39118^{T} strains can adsorb the compound on their biomass, while the liquid phase residue was not cytotoxic in the chronic *Aliivibrio fischeri* test.

6. SCIENTIFIC PUBLICATIONS RELATED TO THE TOPIC OF THE DISSERTATION

Publications in foreign languages in peer-reviewed, scientific journals:

HÁHN, J., SZOBOSZLAY, S., **TÓTH, G.,** KRISZT, B. (2017): Assessment of bacterial biodetoxification of herbicide atrazine using *Aliivibrio fischeri* cytotoxicity assay with prolonged contact time. *Ecotoxicology*, 26:648-657. https://doi.org/10.1007/s10646-017-1797-0 **IF= 1,951; Q2**

KASZAB, E., RADÓ, J., KRISZT, B., PÁSZTI, J., LESINSZKI, V., SZABÓ, Á., **TÓTH, G.,** KHALEDI, A., SZOBOSZLAY, S. (2019): Groundwater, soil and compost, as possible sources of virulent and antibiotic-resistant *Pseudomonas aeruginosa*. *International Journal Of Environmental Health Research*, 1691719, 13. https://doi.org/10.1080/09603123.2019.1691719 **IF=1,916; Q3**

TÓTH, G., HÁHN, J., KRISZT, B., SZOBOSZLAY, S. (2019): Acute and chronic toxicity of herbicides and their mixtures measured by *Aliivibrio fischeri* ecotoxicological assay. *Ecotoxicology and Environmental Safety*, 185, 109702. https://doi.org/10.1016/j.ecoenv.2019.109702 **IF=4,872; Q1.**

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Cumulative impact factor: 18,998

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TÓTH, G., HÁHN, J., SZOBOSZLAY, S., KRIFATON, CS., RADÓ, J., KRISZT, B. (2015): Investigation of the biodegradation and cytotoxicity of various herbicides. *ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA*, 62:(Suppl.2) pp. 232-233. 17th International Congress of the Hungarian Society for Microbiology. Budapest, Magyarország.

TÓTH, G., HÁHN, J., SZOBOSZLAY, S., KRISZT, B. (2016): Investigation of the biodegradation products of herbicides by chronic *Aliivibrio fischeri* test. *A Magyar Mikrobiológiai Társaság 2016. évi Nagygyűlése és a XII. Fermentációs Kollokvium*. Absztraktfüzet. Keszthely, 2016. október 19-21., pp. 60-61.

FARKAS, M., RADÓ, J., HÁHN, J., KASZAB, E., **TÓTH, G.,** KRISZT, B., BORDÓS, G., BOKOR, Á., SZOBOSZLAY, S. (2018): Microbial and ecotoxicological monitoring of Lake Balaton and its watershed. *A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium*. Absztraktfüzet,14 p.

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RADÓ, J., SZOBOSZLAY, S., FARKAS, M., **TÓTH, G.,** HÁHN, J., PEER, G., BALÁZS, A., RISA, A., BENEDEK, T., KRISZT, B. (2017): A Balaton vízgyűjtő mikrobiológiai és ökotoxikológiai vizsgálata (1). *VII. Ökotoxikológiai konferencia előadás és poszter kötete*. p. 31., 2017. november 24., Budapest, ISBN 978-963-89452-8-0.

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