IN VIVO AND *IN VITRO* TESTS FOR THE DETECTION OF BIOCHEMICAL AND ECOTOXICOLOGICAL EFFECTS OF THE HERBICIDE ACTIVE INGREDIENT GLYPHOSATE

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Abstract

Aquatic organisms are outstandingly exposed to water contaminants because of their unavoidable contact with xenobiotics, thus their exposure needs to be routinely monitored. Due to its extensive use, the herbicidal agrochemical active ingredient glyphosate realizes massive exposure, its toxic effects alone and in formulations were evaluated in different *in vivo* aquatic ecotoxicological tests on various algae species, freshwater biofilm communities, *Daphnia magna*, and *Danio rerio*, furthermore the possible cytotoxic, genotoxic, and hormone-modulating effects were evaluated *in vitro* on different cell lines and test organisms. Significant differences were detected in the individual and combined toxicity of glyphosate and its co-formulants presented in the formulations, therefore various additives cannot be classified as unequivocally inactive components. The result of the *in vivo* testing proved higher toxicity for the formulating agent and the formulation compared to the individual effect of glyphosate, and significant differences in the sensitivity of test species, and the effects on the sexual development of fish were also observed. The performed *in vitro* assays on cell lines demonstrated the potential cytotoxic and genotoxic effects of glyphosate and its formulations, and some of the effects are the result of the individual toxicity of glyphosate.

Introduction

The main part of pesticide active ingredients (e.g., glyphosate), and their formulations exert an increased load on our environment [1-3]. Plant protection products contain various additives (e.g., formulating agents) in order to enhance the effectivity and bioavailability of the formulation, and these components have long been considered as *inert* ingredients from the aspect of the required main biological effect of the formulation, although co-formulants may have a possible adverse effect on the non-target organisms [4,5]. The occurrence of the market-leading, non-selective herbicide active ingredient and desiccant glyphosate in environmental matrices (e.g. surface water) is globally observed, and these residues may exert harmful effects on non-target organisms, while the biologically active compounds can interact with the abiotic and biotic elements of the ecosystems. Our research group has been working for over a decade now on the evaluation of the ecotoxicity of glyphosate and its formulation. The main purpose of the work has been a systematic evaluation of the ecotoxicity concerns about glyphosate-based formulations carried out directly by testing of the active ingredient and the formulating agent or indirectly by comparing the biological effects of glyphosate and its formulated products. Ecotoxicological evaluation targeted on lethal or sublethal effects e.g., immobilization, algal growth inhibition, phytotoxic, cytotoxic, genotoxic, or hormonal effects tested in *in vitro* assays on cell cultures or in *in vivo* biotests on indicator organisms.

Experimental

The lethal and sublethal effects of glyphosate and its formulations were determined in different standard *in vivo* ecotoxicological standard test methods on various aquatic indicators, as the exposure of aquatic organisms to xenobiotics is unavoidable in surface waters. During our measurements the effects on the immobility of *Daphnia magna*, the growth of various algae species (unicellular floating green algae and cyanobacteria species), moreover the teratogenic effects on *Danio rerio* embryos were also evaluated based on the related OECD guidelines. Furthermore, the effects of glyphosate and its formulation (ROUNDUP CLASSIC) were assessed on the biomass and composition of algal communities in natural freshwater biofilm at the community level as well, completed with a glyphosate degradation test. Before the biofilm testing, freshwater biofilms were grown on glass substrates with the use of a special buoy developed for biofilm colonization under natural conditions in Hungarian river and standing water bodies.

The possible cytotoxic effects of glyphosate were determined *in vitro* by the evaluation of the effects on cell viability, apoptosis, cell cycle, and barrier function. Cytotoxicity of glyphosate IPA salt, its formulations, and POEA was investigated on NE-4C neuroectodermal, MC3T3-E1 preosteoblast, JEG3 placenta choriocarcinoma, and IPEC-J2 porcine intestinal columnar epithelial 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, Muse Cell Analyzer flow cytometer using Muse Caspase 3/7 and Muse Cell Cycle Assay kits, while cell integrity was visualized by holographic transmission microscopy, while barrier functions of IPEC-J2 cells were determined by transepithelial electrical resistance. Furthermore, the effects of glyphosate on cell adhesion via Arg-Gly-Asp (RGD)-dependent integrins were assessed with the use of label-free optical biosensing and ELISA methods. Genotoxic effects of the components of 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 on human HepG2, NE-4C, and MC3T3-E1 cell lines. Moreover, SOS-Chromotests were performed on *E. coli* PQ37 strain. The test measures the primary response of a cell to genetic damage. Briefly it detects *sos* error prone of the *sfiA* gene induced by most genotoxins and it is coupled to a gene that expresses a colorimetric endpoint. Studies also targeted on hormonal modulation and effects on sexual development by the *in vitro* determination of aromatase activity on JEG3 cell lines, while endocrine disrupting effects of the components in ROUNDUP CLASSIC were investigated also *in vivo* on *D. rerio*.

Results and discussion

Based on the results of acute immobilization tests performed on our own laboratory *D. magna* colony and on juveniles ensured by the Daphtoxkit F test kit (MicroBioTests), the order of toxicity for both tested *D. magna* strains and glyphosate-based formulations (ROUNDUP CLASSIC and MEDALLON PREMIUM) was as follows: active ingredient < formulations < formulating agents. In the algal growth inhibition tests, the order of toxicity mostly agreed with the tendency observed for *D. magna* on the basis of the calculated 72h EC_{50} values according to the result of optical density and chlorophyll-a-content measurements. The results of the algal assays indicated significant differences in the sensitivity of different algae species. The photosynthetic activity of *Pseudokirchneriella subcapitata* green algae was significantly decreased after the ROUNDUP CLASSIC exposure but only at the highest investigated concentration (18,9 mg/l POEA and 50,6 mg/l glyphosate), while photochemical efficiency of the PS II photochemical system was not affected by the exposure of glyphosate and POEA alone. During the determination of lethal and sublethal teratogenic effects of glyphosate, its formulations (ROUNDUP CLASSIC, MEDALLON PREMIUM, TOTAL, and GLYPHOS), and

formulating agents (POEA, POE alkyl phosphate ester, sodium-alkyl polyglucoside citrate, and sulfosuccinate) on *D. rerio* embryos, glyphosate was the least toxic and the formulating agent POEA showed the highest toxicity also on embryos. The highest toxicity was observed for ROUNDUP CLASSIC during the testing of the formulations. Below or near the calculated LC_{50} values, deformities, edema (pericardial), inhibition of heartbeat and circulation were the most frequently observed teratogenic malformations in every treatment. Based on the analytical determination of retinoids responsible for the differentiation, development, and embryogenesis of vertebrates, formulating agents used in glyphosate-based herbicides and the active ingredient itself has been indicated to interfere with the formation of retinoids and the retinoid acid pathway [6]. Based on the results of biofilm testing, significant differences were observed in the sensitivity of biofilms grown under different natural conditions. In glyphosate-treated biofilms (100 µg/l glyphosate a.i.), an increase of algal biomass was detected, although in some cases only after an initial decrease, while usually a decrease in the algal biomass was detected in ROUNDUP CLASSIC-exposed biofilms. Individual POEA treatment resulted in the increase of biomass values, however, in samples from Lake Velencei, but only after an initial decrease similar to the effects of glyphosate. The realignment of algal communities was detected in the treated biofilms compared to the control units, while more sensitive species were replaced by more tolerant especially filamentous green algae species, which can utilize the tested substances as a source of nutrients. At the end of the testing period, increased production of *EPS*-matrix (extracellular, mucous exopolymers) was demonstrated in the treated biofilms, especially in the presence of POEA, which can be interpreted in biofilms as an intensified stress response [7]. The result of the trait-based analysis of diatom communities' biological characteristics, significant changes were detected in the cell number ratio and ecological guild categories of the diatom communities in the treated biofilms. Based on our degradation study, the dissipation of glyphosate was highly depended on the form of glyphosate (pure active ingredient or formulated glyphosate), the presence of POEA or biofilms, and the physical/chemical characteristics of surface waters (e.g., pH, the composition of the microbial community) [7].

According to the result of cytotoxicity tests, ROUNDUP CLASSIC significantly decreased the viability of NE-4C cells, while the inhibitory effect of POEA was detected on cellular metabolism. Based on the MTT-assays performed on NE-4C and MC3T3-E1, the order of cytotoxicity was the following: glyphosate IPA salt < ROUNDUP CLASSIC < POEA. The results of the determination of the ratio of total apoptotic cells, a significantly higher level of apoptotic cells was detected for POEA compared to glyphosate IPA and ROUNDUP CLASSIC. The distribution of cells 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. After 24 hrs, for all test compounds ratio of cells in the beginning DNA replicating (S) phase decreased. After the 48-hr exposition, this decrease was more outstanding, moreover ratio of cells in the growth (G_0/G_1) phase increased compared to the control [8,9]. Moreover, NE-4C cells showed a higher sensitivity for the effects of the tested compounds compared to the MC3T3-E1 cell line, and the order of the inhibitory potency was as follows: glyphosate IPA salt < < ROUNDUP CLASSIC < POEA [10]. Based on the detection of cell toxicity by label-free Epic BenchTop optical biosensor, a higher ratio of apoptotic cells was detected in cells exposed to POEA and ROUNDUP CLASSIC compared to glyphosate-treated cells at equivalent concentrations indicating higher apoptosis-inducing potential of POEA [11]. 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. Due to the treatments, a time-dependent decrease in cell area and an increase in the maximum thickness of the NE-4C cells were demonstrated, however, the difference was statistically significant for glyphosate treatment. 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 CLASSIC, practically equitoxic with each other at concentrations 20-fold below agricultural application [8,9]. Based on the assessment of the cytotoxic effects on human JEG3 placenta choriocarcinoma cell lines, all the tested formulating agents and formulations were comparably cytotoxic well below the agricultural dilution of 1%, while the cytotoxic effect of glyphosate was not demonstrated. During the assessment of the individual and combined effects of glyphosate and POEA on cell barrier functions, the 2-hr exposition of POEA and ROUNDUP CLASSIC increased the paracellular integrity of IPEC-J2 cells [12,13], but were toxic to various cancer cells [14]. Furthermore, the effects of glyphosate on cellular interactions via Arg-Gly-Asp (RGD)-dependent integrins were demonstrated, [15], and total inhibition of αvβ3 binding to RGD was observed for glyphosate and its primary metabolite (AMPA), and on α5β1 binding to RGD for acetylglycine [16].

Potential genotoxic effects of the investigated components were observed on the investigated cell lines based on the results of the performed Comet assays, while the results of SOS-Chromotests indicated no genotoxicity in the concentration range of 0.03–710 nM. The results of the measurements of the endocrine-disrupting effects of glyphosate IPA salt, glyphosate-based herbicides (ROUNDUP CLASSIC, ROUNDUP WEATHERMax, GLYFOS, KAPAZIN, TOTAL, MEDALLON PREMIUM), and co-formulants (POEA, POE alkyl phosphate ester, alkyl polyglucoside, quaternary ammonium compound) on JEG3 cell line demonstrated decreased aromatase activity both by the individual exposure of co-formulants (POEA and alkyl polyglucoside) and the formulations at an 800-fold lower concentration than the agricultural dilutions, while glyphosate exerted an effect only at 1/3 of the agricultural dilution demonstrating, that the endocrine-disrupting effects of the formulations primarily caused by the presence of the formulating agent [17]. During the *in vivo* assessment of the endocrine-disrupting effects of the components in ROUNDUP CLASSIC (glyphosate and POEA) *D. rerio*, hermaphroditic zebrafish were not presented in either treatment group, while the number of females was higher in glyphosate and POEA treatments after the 20-week exposition.

Besides our work on the embryotoxicity of glyphosate, its formulant and formulations on *D. rerio* [6], *in vivo* effects of these substances have been tested on an ecologically relevant earthworm species (*Lumbricus terrestris*) [18,19]. The experiments demonstrated reduced activity of earthworms upon exposure to commercial glyphosate formulations or the pure active ingredients at concentrations of 40–90 ng/g soil.

Conclusion

Co-formulants presented in the various plant protection products have long been considered as inert components, although these substances can 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, therefore the toxicological and ecotoxicological evaluation of additives is essential during the environmental risk assessment of pesticide formulations applied in agricultural practice. Our results demonstrated altered toxicity of glyphosate-based formulations compared to the individual effect of glyphosate, and the identified biochemical and (eco)toxicological effects including cytotoxicity (on cell lines of epithelial, neural, and other tissues, as well as tumor cells), endocrine-disrupting effects, as well as aquatic ecotoxicity regarded to the presence of the formulating agents, although glyphosate-specific effects were identified during the measurement of RGD-specific cell adhesion.

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