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Cytotoxicity and hormonal activity of glyphosate-based herbicides☆

Gergő Tóth [a], Judit Háhn [b, *], Júlia Radó [a], Diána A. Szalai [a], Balázs Kriszt [a], Sándor Szoboszlay [a]

[a] Szent István University, Faculty of Agricultural and Environmental Sciences, Department of Environmental Protection and Safety, 1 Páter Károly Street, Gödöllő, 2100, Hungary
[b] Szent István University, Regional University Centre of Excellence, 1 Páter Károly Street, Gödöllő, 2100, Hungary

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ABSTRACT

Glyphosate-based herbicides (GBHs) are the most widely used pesticides for weed control. In parallel with the renewal of the active ingredient, polyethoxylated POE(15) containing GBHs were banned in the EU in 2016. Since then, co-formulants were changed and numerous GBHs are marketed with different excipients declared as inert substances. In our study, we focused to determine acute and chronic cytotoxicity (by *Aliivibrio fischeri* assay) and direct hormonal activity (estrogenic and androgenic effects measured by *Saccharomyces cerevisiae* BLYES/BLYAS strains, respectively) of glyphosate, AMPA, polyethoxylated POE(15) and 13 GBHs from which 11 formulations do not contain polyethoxylated POE(15). Among the pure substances, neither glyphosate nor AMPA had any effects, while polyethoxylated POE(15) exhibited pronounced toxicity and was also estrogenic but not androgenic. Regarding the acute and chronic cytotoxicity and hormonal activity of GBHs, dilution percentages calculated from EC_{50} values were in the most cases by one or two order of magnitude lower than the minimum recommended dilution for agricultural and household use. Relation could not be observed between the biological effects and type of glyphosate-salts; hence toxicity could be linked to the co-formulants, which are not even declared in 3 GBHs. Toxicological evaluation must focus on these substances and free accessibility of GBHs should be reconsidered.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine], an amino acid glycine derivative that firstly synthesized in 1950, is a systemic, non-selective organophosphorus herbicide. Originally, it is used for pre- and post-emergent weed control in arable crops, along with wheat, maize and oats. In addition, it is also used to desiccate a wide range of other crops (soybean, cotton, tobacco and GM crops) (Woodburn, 2000). Glyphosate is the most widely used broad-spectrum herbicide with the greatest usage of genetically modified glyphosate-resistant (GR) crops, especially in the USA (Benbrook, 2016). Glyphosate interrupts the metabolic pathway of shikimic acid, which is responsible for the biosynthesis of aromatic amino acids in plants and some microorganisms. It prevents the enzyme that catalyses the formation of the central intermediate, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), by attaching the phosphoenol-pyruvate as its analogue to the substrate of the enzyme. This metabolic pathway inhibits the synthesis of tryptophan, phenylalanine, tyrosine, therefore additional amino acid production is not possible (Orcaray et al., 2010; Steinrücken and Amrhein, 1980). The EPSPS enzyme and the family of genes (*ARO*) responsible for its production are also found in most prokaryotes and lower-class eukaryotes (such as *Saccharomyces cerevisiae*). Thereby, glyphosate could also be used as inhibitors of microbial growth (Braus, 1991; LaRossa and Falco, 1984). Moreover, there are experimental evidences, that glyphosate can inhibit the EPSPS enzyme in the human gut microbiome, modifying the composition of microorganisms, thereby alter the function of the microbiome (Mesnage and Antoniou, 2020; Rueda-Ruzafa et al., 2019). Moreover, in the case of male Sprague-Dawley rats, Roundup® Grand Travaux Plus caused an alteration in the microbiome at environmental concentrations (Lozano et al., 2018). Toxicokinetic studies confirm that glyphosate has low bioavailability and it has slowly and poorly absorbed in the gastrointestinal tract of rats with

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* Corresponding author.
E-mail addresses: toth.gergo@mkk.szie.hu (G. Tóth), hahn.judit@mkk.szie.hu (J. Háhn), rado.julia@mkk.szie.hu (J. Radó), kriszt.balazs@mkk.szie.hu (B. Kriszt), szoboszlay.sandor@mkk.szie.hu (S. Szoboszlay).

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relatively low absorption half-life, although the production of major metabolite (aminomethyl phosphonic acid - AMPA) could have been converted by the microbiome action (Anadón et al., 2009).

Salts of glyphosate have a water solubility up to one or two orders of magnitude higher than that of the free phosphonic acid (10.5 g/L at 20 °C). The order of the water solubility: trimethyl sulfonium salt ≅ isopropyl ammonium salt > potassium salt > sodium salt > ammonium salt > glyphosate, so the active substance with rainwater also reaches deeper layers of the soil, although it undergoes rapid decomposition and strong complexing processes under certain conditions. AMPA is the primary transformation product in plants, water and soil, which is much more mobile in soil than the parent compound (Székács and Darvas, 2012). Glyphosate and AMPA were found in surface waters at relatively higher concentrations (0.12–0.98 μg/L and 2.8 μg/L) above the maximum acceptable level at 0.1 μg/L established by the Drinking Water Directive 98/83/EC (Mörtl et al., 2013; Popp et al., 2008). Over the years, the presence of glyphosate and AMPA in surface- and groundwaters surrounding agricultural, horticultural or viticulture catchment areas may also be more critical after application due to rainfall-run-off events and spray drifting with increasing concentrations (glyphosate: 17–387 μg/L; AMPA: 4.5–49.4 μg/L) (Solomon et al., 2007; Lefrancq et al., 2017; Lutri et al., 2020; Mac Loughlin et al., 2020). According to Cassault-Meyer et al. (2014) and Teleken et al. (2019) GBH formulations at a concentration of 0.5% were found in the environment (surface- and groundwater) after their agricultural usage. By the decision of the European Union in 2017, authorisation for the application of glyphosate as an active substance in PPPs is approved until 15 December 2022 (European Commission, 2017).

For the active substance glyphosate, adhesion and absorption were initially enhanced with tallow amine derivatives, among them a group of compounds called polyethoxylated tallow amine (POEA) is widespread. However, it has been revealed that the side effects of these formulated products are more significant than that of the pure active ingredient. Tsui and Chu (2003) have been demonstrated the toxic effects of glyphosate, glyphosate-formulated product of Roundup® and their most common co-formulant polyethoxylated tallow amine (POEA) on various model species selected from aquatic ecosystems i.e. bacteria (*Aliivibrio fischeri*), microalgae (*Selenastrum capricornutum*, *Skeletonema costatum*), protozoa (*Tetrahymena pyriformis*, *Euplotes vannus*) and crustaceans (*Ceriodaphnia dubia, Acartia tonsa*). Based on the results, POEA was found to be the most toxic compound. Teratogenic effects of glyphosate-based herbicides (GBHs) have also been investigated on *Xenopus laevis* and *Gallus domesticus* embryos. *X. laevis* embryos have become similar phenotypes: the torso and head size have shortened, the eyes have not developed properly or at all (*microphthalmia*), and other skull deformities have developed at later stages of development (Paganelli et al., 2010). Examining the chronic exposure to *Rana pipiens* tadpoles, it was found that, in addition to developmental abnormalities, gonads of 15–20% of them had an abnormal developmental pattern and showed intersexuality by glyphosate formulations containing POEA (Howe et al., 2004).

Glyphosate can provoke neurotoxicity by a dose-related manner in Wistar rats modulating the serotoninergic, dopaminergic, and noradrenergic system. Besides glyphosate, AMPA also has ability to induce cytotoxic effects on neuronal development system throughout oxidative stress and cell death on human neuroblastoma SH-SY5Y cells (Martínez et al., 2018; Martínez et al., 2020).

Many *in vitro* and *in vivo* studies have examined the endocrine-disrupting activity of glyphosate and GBHs, especially on animal/human cell lines. Steroidogenesis inhibition was observed on MA-10 Leydig tumor cell line with disrupting StAR protein expression after Roundup® exposure. It also has induced apoptosis and necrosis in embryonic kidney 293 and choriocarcinoma-derived placental JEG3 cell lines, inhibited the transcriptional activities on transfected-HepG2 and endocrine disruption effect have appeared on the androgen receptor in the breast cancer MDA-MB453-kb2 cell lines (Benachour and Séralini, 2009; Gasnier et al., 2009; Walsh et al., 2000). In the recent years, several other studies indicated endocrine-disrupting effects of glyphosate and GBHs by inducing estrogenic activity with altering ERα and β expression, increasing the expression of an estrogen response element-luciferase reported gene (ERE-luc) in T47D human hormone-dependent breast cancer cells (Mesnage et al., 2017;Thongprakaisang et al., 2013). Roundup® disrupted in the synthesis of the human hormone progesterone by decreasing its production and changing levels of the hormone (Walsh et al., 2000). According to Romano et al. (2010), Roundup® Transorb caused alterations in biosynthesis of testosterone in Wistar rats, but not in levels of estradiol. Two formulations of Roundup® inhibited the production of progesterone in human chorioplacental JAr cells (Young et al., 2015). Lower levels of testosterone and corticosteroids were recorded in male rats, together with reduced sperm count and altered morphology in the testicles as well (Nardi et al., 2017; Pandey and Rudraiah, 2015). In female rats, there were differences in the mammary gland (increased estrogen receptor of ESR1) and ovarian development (decreased cell division), coinciding with lower estradiol levels (Altamirano et al., 2018). Teleken et al. (2019) described that treatment with Roundup® Original DI after pregnancy and lactation in adult female mice caused reduced fertility in male F1 offspring as a maternal effect. Roundup® Original DI has a hormone modulating effect throughout the adult female mice, which is responsible for the partial infertility of the F1 offspring. Recent studies categorized glyphosate and GBHs (especially Roundup® formulations) as endocrine-disrupting chemicals (EDCs) due to their effects on the hormonal system (Gasnier et al., 2009; Richard et al., 2005; Manservisi et al., 2019). Glyphosate, GBHs and co-formulants (including POEA and APG - alkyl polyglucoside) aromatase enzyme inhibitory activity was also noticed from concentrations downward than the agricultural dilutions (Defarge et al., 2016).

Moreover, additive and synergistic effects between glyphosate and its adjuvants, co-formulants and detergents have been observed (Niedobová et al., 2019). In contrast, according to the European Food Safety Authority (EFSA) and U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (ESDP) due to lack of information, there is no convincing evidence of potential interactions with the endocrine system regarding with estrogen and/or androgen signalling pathways (European Food Safety Authority, 2017; US EPA, OCSPP, 2015).

Nowadays (since 2016), the tallow amine group is being replaced for numerous other so-called 'inert' ingredients in GBHs marketed in the European Union; for which, according to the relevant Commission Regulation (EC no. 1107/2009), toxicological evaluation is not required. After ethoxylated amines (family of POEA surfactants), GBHs are formulated with alkylpolyglycosides, non-alkoxylated surfactants, nitrolyl, propoxylated quaternary ammonium surfactant, ethoxylated ether amines and other surfactants or detergent-like substances, which are often not declared precisely by manufacturers, to increase stability and adhering, and improve penetration to the cells, thus bioavailability as well. Hungary has been the leading supplier of 24 POEA-containing glyphosate formulations until withdrawal in 2016. With the simultaneous protection of the active ingredient glyphosate which has an economic aspect, Hungary has recognized the legitimacy of the toxicological criticism of the products (Benachour and Séralini, 2009; Mesnage et al., 2019 NFCSO National Food Security Office,

2016; Székács and Darvas, 2018; Székács et al., 2014; Vandenberg et al., 2017). The European Union's environmental decision-makers have to revise the range of endocrine-disrupting chemicals (EDCs), which are causing a number of critically harmful environmental events (the development of hermaphrodite in male frogs) and disease (male infertility, endometriosis, hormone-dependent cancers). The consequences of the decision will seriously affect the participants of the chemical industry that producing commercial products of pesticides. By June 2020, EDCs should be removed from the European market (Pesticide Action Network, 2019).

Our research was focused to evaluate the acute and chronic cytotoxicity and direct estrogenic and androgenic activity of glyphosate, AMPA, POE(15) and thirteen glyphosate-based herbicides from which eleven formulations do not contain POE(15) and are authorized in Hungary. They are also belonging to the distribution category III., i.e. freely available to anyone without any qualification or licence.

2. Materials and methods

2.1. Stock solutions of glyphosate, AMPA and diluted commercial formulations

Glyphosate, [(N-(phosphonomethyl) glycine)], chemical formula: $C_3H_8NO_5P$, Pestanal®, analytical standard, CAS 1071-83-6) and a major metabolite of glyphosate, AMPA (aminomethyl phosphonic acid), chemical formula: CH_6NO_3P, analytical standard, CAS 1066-51-9, purity 99.9%), polyethoxylated tallow amine − POEA with an average ethoxylation of 15 carbons [POE(15) tallow amine, chemical formula: R-N$(CH_2CH_2O)H_m(CH_2CH_2O)H_n$, CAS 61791-26-2, purity 100%, Greyhound Chromatography and Allied Chemicals], 17β-estradiol (E2, chemical formula: $C_{18}H_{24}O_2$, CAS: 50-28-2, purity ≥98%), 5α-dihydrotestosterone (DHT, chemical formula: $C_{19}H_{30}O_2$, CAS: 521-18-6, purity ≥99%) studied in this work were purchased from Sigma-Aldrich Ltd., Hungary. For experiments, 1.0 mg/mL glyphosate and AMPA stock solutions were prepared from dry compounds dissolved in sterile distilled water. Liquid POE(15) was dissolved also in sterile distilled water to prepare 10 mg/mL stock solution.

GBHs examined in this study according to Table 1., are commercially available in Hungary, unless otherwise are indicated. Barclay Gallup Biograde 360® (Barclay Chemicals (R&D) Ltd., 04.2/5211-1/2015, Hungary), Boom Efekt® (Pinus TKI, 02.5/1146/1/2008, Hungary), Dominator Extra 608 SL® (Albaugh UK Ltd., 04.2/4156-1/2012, Hungary), Fozat 480® (Agro-Chemie Kft., 02.5/11609-1/2010, Hungary), Gladiator 480 SL® (Makhteshim Agan Zrt., withdrawn authorisation in Hungary), Glialka Express 6H® (Monsanto/Bayer, 02.5/11125-2/2010, Hungary), Glialka Star® (Monsanto Europe S.A., 02.5/117/1/2009, Hungary), Glyfos Dakar® (Cheminova A/S, 02.5/1659/4/2009, Hungary), Kapazin® (Arysta LifeSience S.A.S., 02.5/12062-2/2010, Hungary), Medallon Premium® (Syngenta Crop Protection A.G., 02.5/10506-2/2010, Hungary), Roundup® Classic (Monsanto Europe S.A., withdrawn authorisation in Hungary), Roundup® Mega (Monsanto Europe S.A., 02.5/10493-1/2010, Hungary) and Total® (Cresco Chemical Kft., 02.5/12059-2/2010, Hungary) were obtained from Praktika Kft. (2700 Cegléd, Rákóczi street 43., Hungary).

2.2. Acute and chronic cytotoxicity assays

In order to determine the cytotoxicity of glyphosate (G), AMPA, POE(15) and GBHs, standard Microtox® acute assays and chronic tests adapted for microtiter plates have been carried out using *Aliivibrio fischeri* (AVF) (DSM-7151, NRRL B-11177) test organism. *Aliivibrio fischeri* is a bioluminescence marine bacterium which exposed to a toxic chemical reacts by decreased light emission. It is a very sensitive test organism with the ability to indicate any negative changes in the metabolic status of the cell.

The Microtox® acute AVF tests have been performed according to the ISO 11348-2 (2007). 1.0 mg/mL stock solutions of G and AMPA were used and diluted from 5.00E+02 mg/L to 3.00E+00 mg/L, 10 mg/mL stock solution of POE(15) was diluted from 5.00E+02 mg/L to 3.00E+01 mg/L. GBHs were used in their original form to prepare dilution series. After 30 min of exposure, bioluminescence has been measured by Microtox® Model 500 analyser and compared to the initial values. The EC_{50} values given in percentage by Omni™ software (version 1.1, AZUR Environmental Ltd., USA) were recalculated to mg/L based on the initial concentrations of G, G salts, AMPA and POE(15).

Chronic assays with prolonged (25 h) contact time taking in account the diauxic growth of *Aliivibrio fischeri* (Froehner et al., 2002) were performed on flat-bottom PS microtiter plates according to Háhn et al. (2017) with slight modification regarding the preparation of AVF inoculum, which was made by inoculating 50 μL AVF culture stored at −80 °C into 50 mL Bacto Marine Broth (BMB) (Difco 2216), and incubating at 20 °C and at a speed of 170 rpm (Certomat® BS-1, Sartorius Stedim Biotech GmbH, Germany) overnight. Seeing that there are not available relevant literature, several preliminary experiments with roughly estimated effective concentration range have been performed. Based on these results, for this study the chosen concentration range of G was from 5.00E+02 mg/L to 1.00E-03 mg/L, AMPA from 5.00E+02 mg/L to 1.00E-02 mg/L, POE(15) from 1.00E+03 mg/L to 1.00E-04 mg/L. GBHs were used undiluted, the concentrations were tested from 1.00E+01 to 1.00E+06 mg/L of the original G salt content. Sterile distilled water was used as solvent control (SC). After 20 μL of the test materials were dispensed into the wells, 180 μL of the AVF inoculum with the optical density of 0.1 (at a 600 nm wavelength) was pipetted to the samples. Controls, containing only *A. fischeri* cultures were included as well. The applied contact times were 0, 3.5, 10, 15 and 25 h. Bioluminescence was measured by a Victor™ X Light 2030 Luminescence Reader (PerkinElmer, USA). Plates were incubated at 20 °C and at a speed of 250 rpm during experiments in a shaking microplate thermostat (PST-60HL-4, BioSan, Latvia).

2.3. Measurement of direct estrogenic and androgenic activity

To determine the estrogenic, androgenic and cytotoxic effects of the pure G, AMPA, POE(15), and GBHs genetically modified BLYES, BLYAS and BLYR strains of *Saccharomyces cerevisiae* test organism were used. Strains were provided by Eldridge, M. and Sayler, G. from The University of Tennessee (Knoxville, Tennessee, USA). BLYES and BLYAS, containing human estrogen and androgen receptor genes, and plasmids with lux genes beside the respective hormone response elements, have been used to investigate the direct estrogen and androgen receptor binding ability of the test materials by bioluminescence intensification. Constitutive BLYR strain containing only lux genes harbouring plasmids has been applied for measuring cytotoxic effects (Eldridge et al., 2007; Sanseverino et al., 2005, 2009) by decreased light emission.

BLYES, BLYAS and BLYR tests have been carried out as described by Háhn et al. (2016). Briefly: *S. cerevisiae* strains BLYES, BLYAS and BLYR strains were inoculated in uracil and leucine selective minimal medium (Routledge and Sumpter, 1996) to an OD_{600} of 1.0. Pure compounds and formulations were dissolved and diluted in 20 μL sterile, distilled water and 180 μL of yeast were added to the samples.

Similarly to the chronic assay, numerous preliminary experiment have been carried out to determine to effective concentration

Table 1
Chemical properties and ecotoxicity on aquatic organisms of the glyphosate-based herbicides examined in this study based on the Material Safety Data Sheets (MSDS) of the formulations.

Formulation name	Form of the formulation	The concentration of (w/w%) of glyphosate acid (G) as an active substance	Concentration in the formulation (w/w%) of glyphosate salt	Co-formulant(s) (w/w%)	Ecotoxicity of formulations based on the MSDS		Expiration date of the basic licence in Hungary
					Oncorhynchus mykiss (LC_{50} [mg/L], 96 h)	*Daphnia magna* (EC_{50} [mg/L], 48 h)	
Barclay Gallup Biograde 360®	WSC	360 g/L (30.7 ± 1.5%)	485 g/L (30.7 ± 1.5%) IPA salt	not declared	>326	>317	31.12.2021.
Boom Efekt®	WSC	360 g/L (30.8 ± 3.1%)	480 g/L (41.5%) IPA salt	phosphate ester amine salt (5–15%)	>322	>1000	31.12.2021.
Fozat 480®	WSC	360 g/L (30.4%)	480 g/L (41%) IPA salt	not declared	21.6	113.9	15.07.2020.
Gladiator 480 SL®	WSC	360 g/L (30.8%)	480 g/L (41.5%) IPA salt	polyethoxylated tallow amine [POE(15)] (13–18%)	86 (active substance)	780 (active substance)	30.11.2016.
Glialka Express 6H®	RTU	7.2 g/L (0.72 ± 0.11%)	9.6 g/L (1%) IPA salt	pelargonic and related fatty acids (2%) another surfactant, which is not declared (1.5%)	502	323	31.08.2024.
Kapazin®	WSC	360 g/L (30.8%)	486 g/L (41.5%) IPA salt	C8-10 ethoxylated alcohol (<2 g/L) triethylene glycol monobutyl ether (<2 g/L)	>100	>100	01.12.2020.
Roundup® Classic	WSC	360 g/L (30.7%)	486 g/L (41.5%) IPA salt	polyethoxylated tallow amine [POE(15)] (15.5%)	8.2	11	30.11.2016.
Total®	WSC	360 g/L (30.8%)	486 g/L (41.5%) IPA salt	not declared (inert substance)	29.3	76.2	15.12.2020.
Glialka Star®	WSC	360 g/L (28.85 ± 1.44%)	480 g/L (41%) P salt	ethoxylated ether alkylamine (6%)	28	69	31.12.2021.
Roundup® Mega	WSC	450 g/L (34.4%)	551 g/L (42.1%) P salt	ethoxylated ether alkylamine (7%)	28	69	01.07.2020.
Dominator Extra 608 SL®	WSC	480 g/L (39.4 ± 2.1%)	608 g/L (41%) DMA salt	D-Glucopyranose, oligomers, decyl octyl glycosides (<5%), Disodium cocoamphodipropionate (<5%) Methyl alcohol (<1%)	33.1	>120	31.12.2021.
Glyfos Dakar®	G	608 g/kg (68.0 ± 2.5%)	784.4 g/kg (75%) AM salt	ethoxylated hydrogenated fatty acid amine (1–5%)	>100	>100	31.12.2021.
Medallon Premium®	WSC	360 g/L (28.3%)	439 g/L (25–30%) DIAM salt	D-Glucopyranose, oligomers, decyl octyl glycosides (10–20%)	800 (86 – active substance)	160	15.07.2020.

Legend: WSC – Water Soluble Concentrate; G – Granules; RTU – Ready To Use; IPA salt – Isopropylamine salt; DMA salt – Dimethylamine salt; P salt – Potassium salt; AM – Ammonium salt; DIAM – Diammonium salt.

range to measure direct estrogenic and androgenic activity. To widen the range and refine the results, at least 12 dilution members were applied. The exact values for each test material could be found in the supplementary data.

As positive controls, 17β-estradiol (E2) and 5α-dihydrotestosterone (DHT) dissolved in methanol were applied in BLYES and BLYAS test, respectively. The concentration ranges of E2 and DHT were from 1.00E-01 to 4.88E-05 and from 1.00E+00 to 4.88E-04 mg/L. As negative controls, methanol and distilled water were used. Bioluminescence was measured after 5 h, when the bioluminescence response is the most pronounced, by a Victor™ X Light 2030 Luminescence Reader. Experiments were repeated five times, independently.

2.4. Statistical analysis of data

Bioluminescence intensification has been determined as calculated in Froehner et al. (2002) by comparing the average bioluminescence values (CPS) of parallel samples to that of the negative control and were expressed as a percentage. Inhibition (%) values were calculated using the reciprocal of the method mentioned above. In BLYES and BLYAS tests, data were normalized defining the maximum response as 100%. While measuring toxicity by bioluminescence inhibition, maximum response was determined as 100% (total inhibition). Bioluminescence values of the negative controls was used as 0% in every measurement.

Statistical analyses have been performed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). All data are expressed as means and standard deviations. Significant differences with $p \leq 0.05$ between the control and the concentration values were determined using a One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test.

For sigmoidal concentration-response curve fitting, EC_x values calculations and diagrams were generated by four-parametric logistic equation nonlinear regression model (Hill equation or variable slope sigmoidal equation).

3. Results

Cytotoxicity and hormonal activity measured by *Aliivibrio fischeri* and BLYES/BLYAS/BLYR bioreporters are expressed in EC_{50} values. For the better and easier comparison, EC_{50} values of GBHs as

G salts are expressed as well as those of glyphosate acid. Concentration-response curves obtained in the chronic AVF tests and bioreporter assays for each substance and GBHs are provided separately in the supplementary materials.

3.1. Acute and chronic cytotoxicity of glyphosate, AMPA, POE(15) and GBHs

Acute (30 min) and chronic (25 h) toxicity after 30 min and 10 and 15 h of exposure, respectively, measured by bioluminescence inhibition of *Aliivibrio fischeri* are represented in Table 2. Concentration-response curves for the three pure substances [G, AMPA, POE(15)], GBHs with IPA salt and with DMA, P, AM, DIAM salts after 10 and 15 h of contact time are shown in Figs. 1 and 2., respectively, where the co-formulants of the GBHs based on their MSDS are also indicated. After 30 min, glyphosate and AMPA as pure chemicals did not cause bioluminescence inhibition to AVF even at the highest applied concentrations (i.e. their solubility limit). Moreover, a slight elevation in the bioluminescence could be observed at the lowest concentrations in the case of AMPA and POEA; however, the hormesis may be the potential explanation of this phenomenon, i.e. certain toxic agents could be able to result in stimulating or beneficial effects at a low dose (Mattson, 2008). POE(15) has had a toxic effect on AVF, EC_{50} value was 19.3 mg/L. Glialka Express 6H® (IPA salt, ready-to-use formulation) exerted the most toxic effects, where the EC_{50} values were 3.8 and 3 mg/L for the IPA salt and G, respectively. Glialka Express 6H® was followed by Roundup® Classic ($EC_{50\ IPA,\ G}$= 54.7 and 40.5 mg/L), Total® ($EC_{50\ IPA,\ G}$= 73 and 54 mg/L), Roundup® Mega ($EC_{50\ Psalt,\ G}$= 82.7 and 67.5 mg/L), Medallon Premium® ($EC_{50\ DIAM,\ G}$= 88 and 72 mg/L) and Glialka Star® ($EC_{50\ Psalt,\ G}$= 88.2 and 72 mg/L). Toxicity of Roundup® Mega and Glialka Star® was very similar due to their almost identical composition (G salt and co-formulants). The other GBHs were less toxic to *Aliivibrio fischeri* in the acute test: the EC_{50} values were by one order of magnitude higher, from 144 (108 for G) to 606 (445 for G) mg/L.

Regarding the chronic assay, the toxicity of POE(15) was similar than that 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 GBH, with EC_{50} values at 3.9 and 1.2 mg/L after 10 and 15 h 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 h and 26.4 and 30.3 mg/L after 15 h, respectively.

In our previous studies, *Aliivibrio fischeri* had shown the highest sensitivity after 15 h of herbicide exposure in the chronic assay and was more sensitive toward pesticides than in the acute test. After exposure of Glyfos Dakar® ($EC_{50\ 10h,15h,\ AM\ salt}$= 87.8 and 626 mg/L), Barclay Gallup Biograde 360® ($EC_{50\ 10h,15h,\ IPAsalt}$= 4.5 and 26.4 mg/L) and Total® ($EC_{50\ 10h,15h,\ IPAsalt}$= 44 and 196 mg/L), the EC_{50} values were higher at 10 h than that at 15 h. The other GBHs toxicity increased with the time of exposure.

Overall, correlation could not be found between cytotoxicity and the type of glyphosate salt in the GBHs. However, Glialka Express 6H®, the most toxic GBH contains no more than 1% of IPA salt and this is the only formulation with pelargonic acid and related fatty acids as co-formulants. Therefore, the high toxicity is most likely caused by the co-formulants and not by the salt in the IPA containing GBHs.

3.2. Hormonal activity and cytotoxicity of glyphosate, AMPA, POE(15) and GBHs

The direct estrogenic and androgenic activity has been measured by *Saccharomyces cerevisiae* BLYES and BLYAS strains, respectively.

The EC_{50} values (means with standard deviations from the independent bioreporter assays) for the positive control chemicals were 5.93E-10±3.5E+00 M for E2 and 1.79E-08±1.13E+01 M for DHT, respectively, measured in our study. The validation criteria for measurement of direct estrogenic and androgenic activity was sufficient and met the expected literary EC_{50} values, which are 6.3E-10±2.4E-01 M for E2 and 1.1E-08±0.5E-05 M for DHT as positive control chemicals, using BLYES and BLYAS assays, respectively (Sanseverino et al., 2009). Methanol as negative control for E2 and DHT has not resulted in changes in bioluminescence values compared to those of the test organism without any chemical.

Cytotoxicity was checked using the constitutive control BLYR strain. Effective concentrations were expressed referring to the G salt in the formulations and to G as well, in the same way as in the AVF assays (Table 3). Concentration-response curves for the three pure substances [G, AMPA, POE(15)] and GBHs with IPA salt and with DMA, P, AM, DIAM salts after 5 h of contact time are shown in Figs. 3–5., respectively.

Cytotoxicity of the tested formulations can interfere with the detection of the hormonal activity. However, we have performed many preliminary experiments in order to be able to determine precisely whether a formulation or chemical is hormonally active, cytotoxic, or both. If the formulation was cytotoxic in the tested concentration range, the endocrine disrupting effect was also examined in the concentration range one or two orders of magnitude below.

Similarly to the cytotoxicity results, neither G nor AMPA proved to be toxic to BLYR or hormonally active in BLYES and BLYAS assays. POE(15) had cytotoxic effect (EC_{50}= 24 mg/L) and estrogenic activity (EC_{50}= 13.4 mg/L) on BLYR and BLYES, respectively. Among the GBHs Barclay Gallup Biograde 360® (IPA salt), Kapazin® (IPA salt) and Medallon Premium® (DIAM salt) did not cause change in the bioluminescence of any of the bioreporter strains. Glialka Express 6H® (with only 1% of IPA salt and pelargonic acid and other fatty acids as co-formulants), as in the AVF assays, had a strong toxic effect on the *Saccharomyces* strains with very low EC_{50} value (4.5 mg/L). Due to the cytotoxicity, the hormonal activity could not be measured by BLYES or BLYAS. Boom Efekt® (IPA salt) was slightly toxic but had neither estrogenic nor androgenic activity at the non-toxic concentrations. POE(15) containing Gladiator 480 SL® (IPA salt) and Roundup® Classic (IPA salt) alongside with Total® (IPA salt) and Glialka Star® (P-salt) formulated with other co-formulants had moderate toxicity and all of them resulted in bioluminescence intensification in BLYES strain. Glyfos Dakar® (AM salt), formulated with ethoxylated hydrogenated fatty acid amine was the only GBH which had neither cytotoxic effects nor estrogenicity but exhibited androgenic activity (EC_{50}= 22.4 mg/L). Fozat 480® (IPA salt with surfactants not declared), Dominator Extra 608 SL® (DMA salt) and Roundup® Mega (P-salt) proved to be slightly toxic and had not only estrogenic but androgenic activity as well. Observing the standard deviations and concentration-response curve for Glyfos Dakar® in Fig. 5., despite the fairly low R^2 and wide confidence interval, the concentration dependent androgenic effect is clear. Regarding the results from the bioreporter assays, estrogenic and/or androgenic effects cannot be clearly attributed neither the type of G salt nor a well-defined co-formulant of formulations.

However, ready-to-use Glialka Express 6H® containing 1% of IPA salt, 2% of pelargonic acid and other fatty acids and 1.5% of another surfactant, which is a not declared co-formulant, possess cytotoxic effects towards the prokaryotic *Aliivibrio fischeri* and eukaryotic *Saccharomyces cerevisiae* as well, which are unambiguously induced by the co-formulants.

Table 2
Results of the acute (30 min) and chronic (10 h and 15 h) *Aliivibrio fischeri* cytotoxicity tests (EC_{50} [mg/L]).

Pure compounds and formulations	EC_{50} values (mg/L) from acute *A. fischeri* cytotoxicity tests (95% confidence intervals)		EC_{50} values (mg/L) from chronic *A. fischeri* (95% confidence intervals) cytotoxicity tests and R^2 values				Co-formulants in formulation based on the MSDS
	30 min		10 h		15 h		
	G salt	G	G salt	G	G salt	G	
Glyphosate	n.t.		n.d.*		n.d.*		–
AMPA	n.t.		n.d.**		n.d.**		–
POE(15)	19.3		13 (10–17) R^2: 0.979		17 (11–25.7) R^2: 0.947		–
Barclay Gallup Biograde 360® [a]	606 (559–655)	450	4.5 (0.22–93.4) R^2: 0.975	3.4	26.4 (12–58) R^2: 0.970	19.6	not declared
Boom Efekt® [a]	600 (528–672)	450	499 (252–989) R^2: 0.933	374	297 (109–805) R^2: 0.928	222	phosphate ester amine salt (5–15%)
Fozat 480® [a]	144 (144–168)	108	315 (67.9–1464) R^2: 0.900	237	83 (46.3–149) R^2: 0.957	62	not declared
Gladiator 480 SL® [a,f]	144 (144–192)	108	377 (197–720) R^2: 0.963	282	103 (55.7–192) R^2: 0.955	77.6	polyethoxylated tallow amine [POE(15)] (13–18%)
Glialka Express 6H® [a]	3.8 (2.9–4.8)	3	3.9 (2.3–6.8) R^2: 0.956	2.97	1.2 (0.25–6) R^2: 0.952	0.9	pelargonic and related fatty acids (2%), another surfactant, which is not declared (1.5%)
Kapazin® [a]	292 (292–316)	216	300 (87.9–1029) R^2: 0.860	223	130 (75.2–227) R^2: 0.957	96.7	C8-10 ethoxylated alcohol (<2 g/L), triethylene glycol monobutyl ether (<2 g/L)
Roundup® Classic [a,f]	54.7 (36.5–54.7)	40.5	393 (268–578) R^2: 0.961	291	119 (76.9–183) R^2: 0.964	87.7	polyethoxylated tallow amine [POE(15)] (15.5%)
Total® [a]	73 (73–73)	54	44 (15.5–125) R^2: 0.741	32.6	196 (136–283) R^2: 0.943	145	not declared (inert substance)
Dominator Extra 608 SL® [b]	159 (152–167)	126	344 (124–954) R^2: 0.858	271	179 (85.9–371) R^2: 0.890	141	D-Glucopyranose, oligomers, decyl octyl glycosides (<5%) Disodium cocoamphodipropionate (<5%) Methyl alcohol (<1%)
Glialka Star® [c]	88.2 (88.2–110)	72	623 (404–962) R^2: 0.938	509	190 (117–307) R^2:0.945	155	ethoxylated ether alkylamine (6%)
Roundup® Mega [c]	82.7 (82.7–82.7)	67.5	437 (291–655) R^2: 0.965	356	142 (92.3–218) R^2: 0.972	116	ethoxylated ether alkylamine (7%)
Glyfos Dakar® [d]	299 (299–299)	243	87.8 (21.4–361) R^2: 0.581	71.3	626 (411–954) R^2: 0.816	508	ethoxylated hydrogenated fatty acid amine (1–5%)
Medallon Premium® [e]	88 (66–88)	72	39.3 (18.4–84) R^2: 0.921	32.2	30.3 (17.7–51.9) R^2: 0.948	24.8	D-Glucopyranose, oligomers, decyl octyl glycosides (10–20%)

Legend: G salt – glyphosate salt; G – glyphosate acid (converted from G salt content); n = 3 in the acute test (performed based on ISO11348:2) and in the chronic test; n.d.* - not determinable; maximum inhibition values were 43.3 and 36.8% at a concentration of 100 mg/L after 10 h and 15 h of exposure, respectively; n.d.** - not determinable, maximum inhibition values were 23.8% after 10 h and 21.7% after 15 h at a concentration of 10 and 50 mg/L, respectively.

[a] IPA salt.
[b] DMA salt.
[c] P salt.
[d] AM salt.
[e] DIAM salt.
[f] POE(15) containing formulation.

3.3. Cytotoxicity and hormonal activity of GBHs expressed as a percentage of dilution compared to the recommended values for agricultural and household use

Table 4 contains the dilution percentages of formulations converted from mg/L values of effective concentrations for expressing cytotoxicity against *Aliivibrio fischeri* and *Saccharomyces cerevisiae* BLYR and hormonal activity measured by *S. cerevisiae* BLYES and BLYAS, and for comparing those to the recommended dilution for agricultural cultivation and home gardening/household use.

Almost all the GBHs had a cytotoxic effect on *Aliivibrio fischeri* at a dilution of one or two orders of magnitude smaller than the

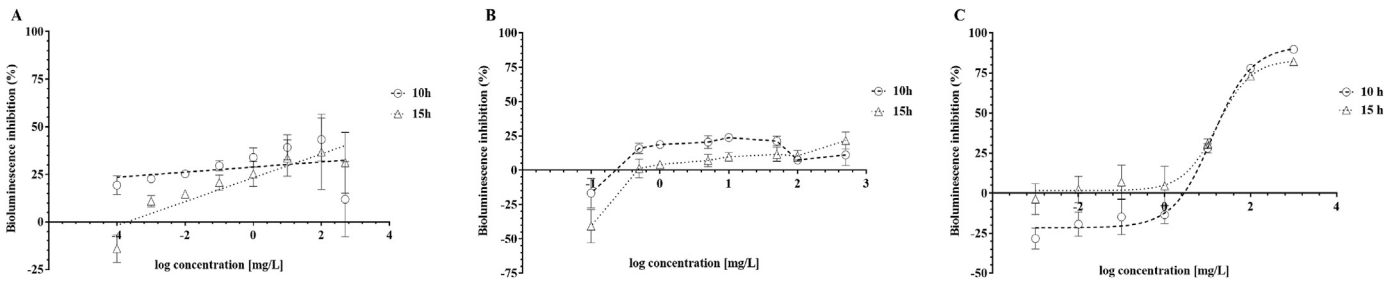

Fig. 1. Chronic cytotoxic effects of the pure compounds (A - glyphosate, B - AMPA, C - POEA [POE(15)]) in *Aliivibrio fischeri* bioluminescence inhibition assay measured after 10 h and 15 h of exposure. Bioluminescence inhibition data are expressed as mean ± SD compared to the control (0%).

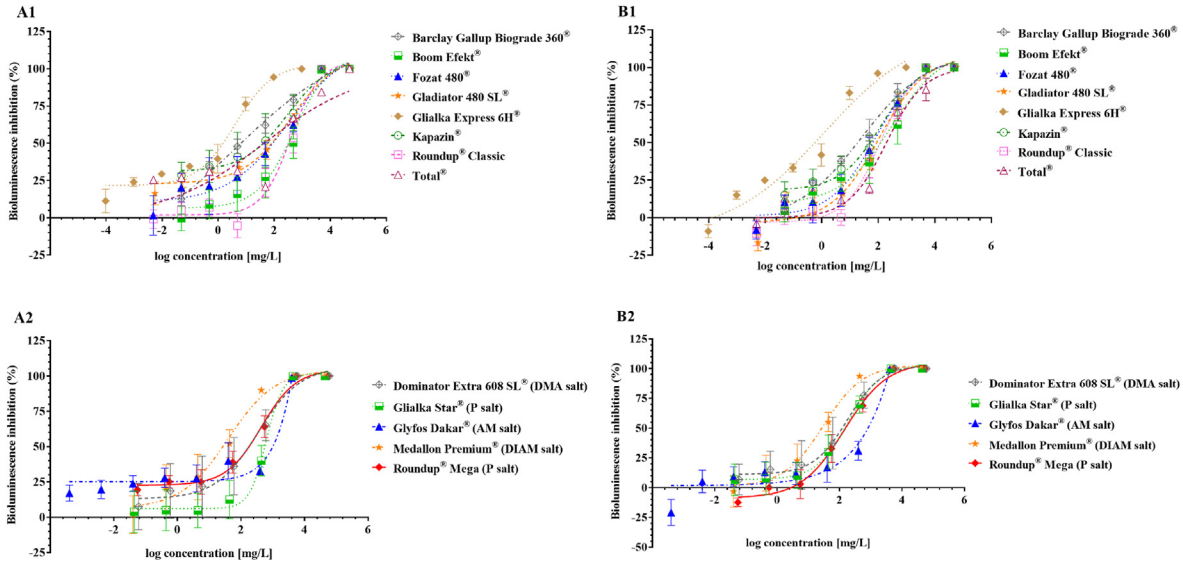

Fig. 2. Chronic cytotoxic effects of the GBHs in *Aliivibrio fischeri* bioluminescence inhibition assay measured after 10 h (A1 and A2) and 15 h (B1 and B2) of exposure. Bioluminescence inhibition data are expressed as mean ± SD compared to the control (0%). A1 and B1 - GBHs with IPA salt (Barclay Gallup Biograde 360®, Boom Efekt®, Fozat 480®, Gladiator 480 SL®, Glialka Express 6H®, Kapazin®, Roundup® Classic, Total®); A2 and B2 - GBHs with DMA, P, AM, DIAM salt of glyphosate (Dominator Extra 608 SL®, Glialka Star®, Glyfos Dakar®, Medallon Premium®, Roundup® Mega).

Table 3
Results of the estrogenic and androgenic activity (measured by BLYES and BLYAS strains) and cytotoxicity (measured by BLYR strain) after 5 h (EC_{50} [mg/L]).

Pure compounds and formulations	Estrogenic and androgenic activity EC_{50} values (mg/L), (95% confidence intervals) and R^2 values				Cytotoxicity EC_{50} values (mg/L), (95% confidence intervals) and R^2 values		Co-formulants in formulation based on the MSDS
	BLYES strain 5 h		BLYAS strain 5 h		BLYR strain 5 h		
	G salt	G	G salt	G	G salt	G	
Glyphosate	n.e.a.		n.a.a.		n.t.		–
AMPA	n.e.a.		n.a.a.		n.t.		–
POEA (POE-15)	13.4 (12.8–14) R^2: 0.964		n.a.a.		24 (22–26.4) R^2: 0.945		–
Barclay Gallup Biograde 360® [a]	n.e.a.	n.e.a.	n.a.a.	n.a.a.	n.t.	n.t.	not declared
Boom Efekt® [a]	n.e.a.	n.e.a.	n.a.a.	n.a.a.	7629 (5567–10456) R^2: 0.823	5721	phosphate ester amine salt (5–15%)
Fozat 480® [a]	169 (156–182) R^2: 0.731	127	52 (42.5–63.7) R^2: 0.781	39	1118 (771–1621) R^2: 0.451	839	not declared
Gladiator 480 SL® [a, f]	85.8 (79.1–93.1) R^2: 0.903	64.4	n.a.a.	n.a.a.	490 (454–530) R^2: 0.954	368	polyethoxylated tallowamine [POE(15)] (13–18%)
Glialka Express 6H® [a]	n.e.a.	n.e.a.	n.a.a.	n.a.a.	4.5 (3.4–5.9) R^2: 0.872	3.4	pelargonic and related fatty acids (2%), another surfactant, which is not declared (1.5%)
Kapazin® [a]	n.e.a.	n.e.a.	n.a.a.	n.a.a.	n.t.	n.t.	C8-10 ethoxylated alcohol (<2 g/L), triethylene glycol monobutyl ether (<2 g/L)
Roundup® Classic [a, f]	57.5 (52.8–62.5) R^2: 0.810	42.6	n.a.a.	n.a.a.	104 (102–107) R^2: 0.979	77	polyethoxylated tallowamine [POE(15)] (15.5%)
Total® [a]	72.8 (66.2–80.0) R^2: 0.848	53.9	n.a.a.	n.a.a.	103 (98.2–107) R^2: 0.931	76	not declared (inert substance)
Dominator Extra 608 SL® [b]	248 (163–379) R^2: 0.602	196	72.2 (46.3–113) R^2: 0.546	57	1758 (1647–1877) R^2: 0.962	1389	D-Glucopyranose, oligomers, decyl octyl glycosides (<5%) Disodium cocoamphodipropionate (<5%) Methyl alcohol (<1%)
Glialka Star® [c]	132 (125–140) R^2: 0.810	108	n.a.a.	n.a.a.	132 (127–138) R^2: 0.935	108	ethoxylated ether alkylamine (6%)
Roundup® Mega [c]	127 (123–130) R^2: 0.921	103	3.9 (2.1–7.5) R^2: 0.545	3.2	137 (130–145) R^2: 0.919	111	ethoxylated ether alkylamine (7%)
Glyfos Dakar® [d]	n.e.a.	n.e.a.	22.4 (7.7–65.5) R^2: 0.173	20.2	n.t.	n.t.	ethoxylated hydrogenated fatty acid amine (1–5%)
Medallon Premium® [e]	n.e.a.	n.e.a.	n.a.a.	n.a.a.	n.t.	n.t.	D-Glucopyranose, oligomers, decyl octyl glycosides (10–20%)

Legend: G salt – glyphosate salt; G – glyphosate acid.
[a] IPA salt.
[b] DMA salt.
[c] P salt.
[d] AM salt.
[e] DIAM salt.
[f] POE(15) containing formulation; n.e.a.: no estrogenic activity (intensification in bioluminescence) was found at the tested concentration; n.a.a.: no androgenic activity (intensification in bioluminescence) was found at the tested concentrations; n.t.: non-toxic - toxic effect (inhibition in bioluminescence) was not found at the tested concentrations; n = 3 in the BLYES/BLYAS/BLYR tests at 5 h.

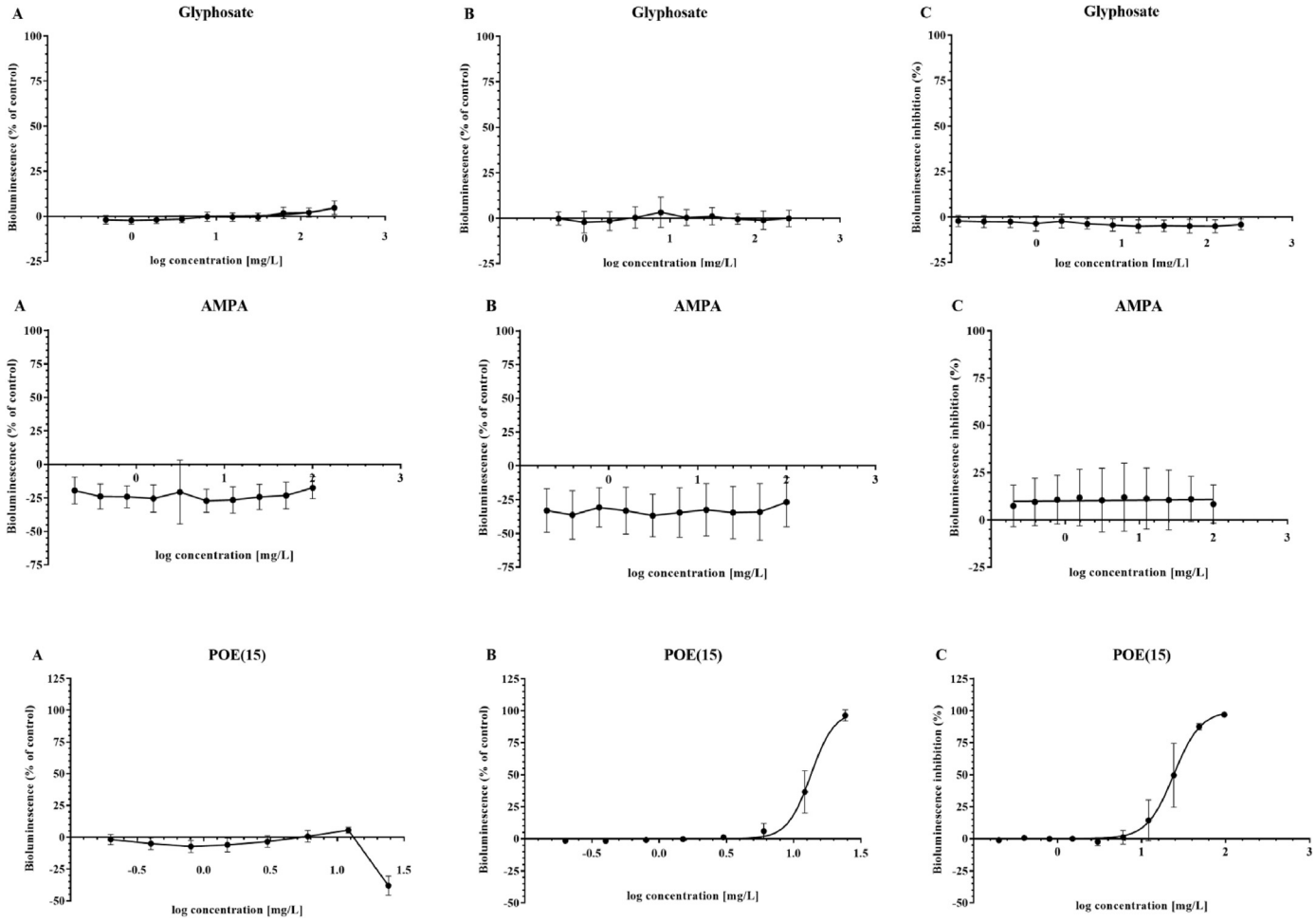

Fig. 3. Androgenic, estrogenic and cytotoxic effects of the pure compounds (glyphosate, AMPA, POEA [POE(15)]) in *S. cerevisiae* bioluminescence bioreporter assays measured by BLYAS (A) BLYES (B), and BLYR (C) after 5 h of contact time. Bioluminescence data are expressed as mean ± SD in percentage of control (0%).

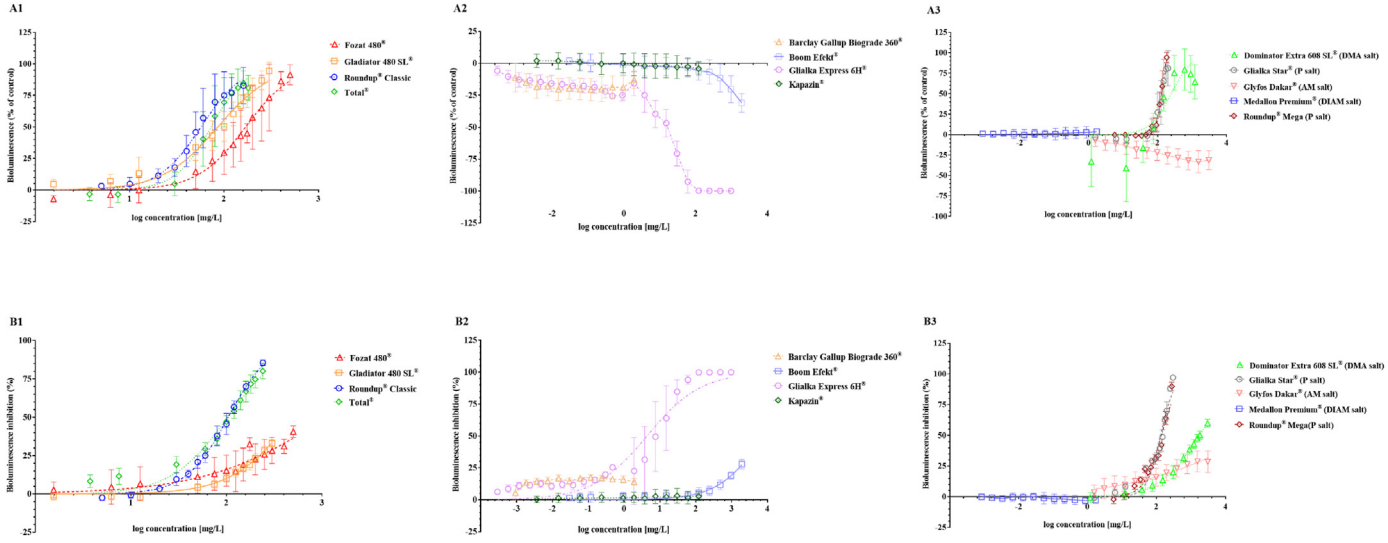

Fig. 4. Estrogenic and cytotoxic effects of the GBHs with IPA salt (Barclay Gallup Biograde 360®, Boom Efekt®, Fozat 480®, Gladiator 480 SL®, Glialka Express 6H®, Kapazin®, Roundup® Classic, Total®) and GBHs with DMA, P, AM, DIAM salt of glyphosate (Dominator Extra 608 SL®, Glialka Star®, Glyfos Dakar®, Medallon Premium®, Roundup® Mega) in *S. cerevisiae* bioluminescence bioreporter assays after 5 h of contact time. A1, A2 – estrogenic activity of GBHs containing IPA salt measured by BLYES; A3 – estrogenic activity of GBHs with DMA, P, AM, DIAM salt measured by BLYES; B1, B2 – cytotoxicity of GBHs containing IPA salt measured by BLYR; B3 – Cytotoxicity of GBHs with DMA, P, AM, DIAM salt measured by BLYR. Bioluminescence data are expressed as mean ± SD in percentage of control (0%).

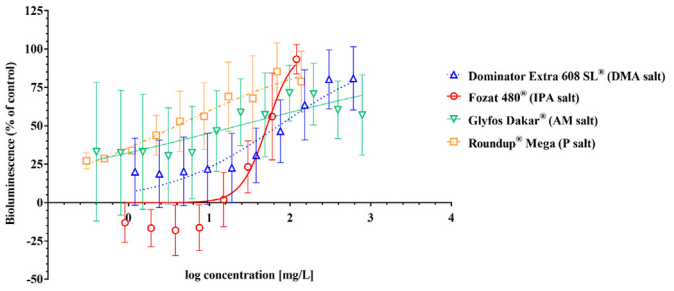

Fig. 5. Androgenic effects of the GBHs (Dominator Extra 608 SL®, Glialka Star®, Glyfos Dakar®, Medallon Premium®, Roundup® Mega) in *S. cerevisiae* bioluminescence bioreporter assays measured by BLYAS (intensification in bioluminescence) after 5 h of contact time. Bioluminescence data are expressed as mean ± SD in percentage of control (0%).

minimum recommended value for agricultural (0.15-3.5%) and household (0.66-2%) use. In the acute assay, Barclay Gallup Biograde 360® and Boom Efekt® proved to be the least toxic, but even half of the lowest recommended dilution of these GBHs resulted in 50% bioluminescence inhibition in *A. fischeri* after 30 minutes of exposure.

A similar phenomenon could be observed when measuring direct hormonal activity; while Medallon Premium® had estrogenic activity at relatively high (16%) dilution, the other GBHs 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 for both agricultural and household use.

4. Discussion

Adverse biological effects of glyphosate-based herbicides had been described over the years. Aromatase altering and other endocrine-disrupting effects and cytotoxicity on cell lines (Cassault-Meyer et al., 2014; Clair et al., 2012; Teleken et al., 2019; Young et al., 2015), genotoxicity and cytotoxicity on fish (Marques et al., 2014; Vera-Candioti et al., 2013), reprotoxicity in *Daphnia magna* (Cuhra et al., 2013) caused by POE(15) containing GBHs had been observed. Recently, Defarge et al. (2018) investigated numerous GBHs without POE(15) and described aromatase activity on JEG-3 cell line and cytotoxicity on HEK 293 cells.

In our study, we focused on the hormone-disrupting and cytotoxic effects of thirteen glyphosate-based herbicides (GBHs) along with glyphosate, AMPA and POE(15). The selected GBHs containing different excipients and glyphosate-salt are permitted in Hungary and the EU except for POE(15)-containing Roundup® Classic and Gladiator 480 SL®.

Here we report the first data of acute and chronic cytotoxic effects on *Aliivibrio fischeri* ecotoxicological test organism caused by GBHs without POE(15). This is also the first *in vitro* results of direct estrogenic and androgenic effects of these formulations.

While the EU policy and legislation on pesticides, as active ingredients are well defined, the authorisation of the so-called “inert” co-formulants are practically non-existent. According to Article 27 of the European Regulation no. 1107/2009, use of co-formulant with a harmful effect on human or animal health, plants, plant-products, or on groundwater and the environment is prohibited. Annex III of the same regulation lists the non-authorized co-formulants; however, the annex does not list any chemicals at the present time. Furthermore, it is generally assumed, that the co-formulants have independently neither significant toxic nor synergistic effects.

According to our findings, all the thirteen investigated GBHs were cytotoxic to *Aliivibrio fischeri* in both acute and chronic tests, and 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 (0.2-3.5% doses in the spray tank) or at concentrations 0.5% that was found in surface and groundwaters after their application (Cassault-Meyer et al., 2014; Teleken et al., 2019). The most pronounced hormonally active effects were induced below the concentration level of 0.5%, in several cases by 2 or 3 orders of magnitude lower. Glyphosate as an active ingredient and AMPA do not have any toxic effects.

Relation of cause and effect could be not observed between the biological effects and the type of glyphosate salt in the GBHs; however, cytotoxicity could be linked to the co-formulants used in the formulations. The most explicit toxicity has been detected after

Table 4
EC_{50} values for cytotoxicity and hormonal activity converted to dilution percent for formulations.

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

Legend.
[a] IPA salt.
[b] DMA salt.
[c] P salt.
[d] AM salt.
[e] DIAM salt.
[f] POE(15) containing formulation; n.e. – no effect; n.m. – not measurable because of cytotoxic effect.

exposure of Glialka Express 6H®, which active ingredient content (1% of IPA salt) is the smallest among the investigated GBHs; however, it is the only formulation in which 2% of pelargonic acid and "other fatty acids" and 1.5% of another unidentified surfactant are the co-formulants. Pelargonic acid, also called nonanoic acid, occurs naturally in plants and animals and is marketed as active substance in herbicides. When applied to plants, it desiccates green tissue, providing rapid and non-selective burn-down. It has low acute toxicity to mammals and birds and is classified as slightly toxic to fish and amphibians; however, as it is used as an antimicrobial (mostly antifungal) agent in food preservation, it inhibits the growth of microorganisms, especially fungi at a low concentration (0.04 mg/L) (MMWD, 2010) which could contribute to the high toxicity of Glialka Express 6H®. Moreover, Glialka Express 6H® is marketed as a ready-to-use product in a spray bottle. It is also concerning, that several GBHs are marketed with not specified co-formulants. In our study, Barclay Gallup Biograde 360®, Fozat 480® and Total® also were investigated. Beside of the acute and chronic cytotoxicity of all of them, Total® exhibited estrogenic activity, while both estrogenic and androgenic activity could be observed caused by Fozat 480®. The active ingredient is IPA salt of glyphosate in all three formulations, although the co-formulants are not declared in either of them.

According to the latest data of the Hungarian National Food Chain Safety Office (NÉBIH/NFCSO), the plant protection products marketed in Hungary exceeded 26 thousand tonnes in 2018, glyphosate as active substance, sold in the highest volume, rounded to 1.334 tonnes. In addition to agriculture, glyphosate is used alongside railway tracks, to forest and shrub control, likewise in households. Plant protection products containing only glyphosate as active substance belong to distribution category III. as freely available products (NFCSO, 2019). Thus, the implementation of good plant protection practice while using glyphosate-based herbicides depends only on following the regulation and directions of the package leaflet. For example, it has been recently published as agricultural workers obtained POE(15) (which is still authorized as a co-formulant in pesticide formulations containing other active ingredients) as a separate adjuvant and mixed with glyphosate formulations in spray tank (Mesnage and Antoniou, 2018).

The hazard classification of the Regulation (EC) No. 1272/2008 on Classification, Labelling and Packaging of Chemicals and Mixtures (CLP) of the European Parliament and Council listed in the decree of 11 of the 13 investigated formulations to the "H411 toxic to aquatic life with long-lasting effects, aquatic chronic 2. class" and 2 of the 13 (Dominator Extra 608 SL®, Total®) to the "H412 harmful to aquatic life with long-lasting effects, aquatic chronic 3. class" according to Material Safety Data Sheets (MSDS). In several cases, the environmental hazards of the formulations were determined by the classification and concentration of the active substance. 3 co-formulants in 5 formulations, namely ethoxylated hydrogenated fatty acid amine [Glyfos Dakar®], ethoxylated ether alkylamine [Glialka Star®, Roundup® Mega], POE(15) [Gladiator 480 SL®, Roundup® Classic], were identified as toxic to aquatic life. However, based on the scientific results that the active substance glyphosate is not carcinogenic, mutagenic or endocrine disrupting chemical, such biological effects were not determined for the formulations and the components of the mixture are not classified based on the regulatory data. Furthermore, data on formulants are not available. EC_{50} values derived from the chronic AVF experiments in our study were compared to those on other aquatic organisms found in the MSDSs. In the case of *Daphnia magna* (Table 1) remarkable differences could be found: EC_{50} values measured by *Aliivibrio fischeri* were by one and two orders of magnitude lower than in *Daphnia magna* 48 h assay for the following formulations – Barclay Gallup Biograde 360®, Boom Efekt®, Fozat 480®, Gladiator 480 SL®, Glialka Express 6H®, Glyfos Dakar®, Medallon Premium®, Total®.

Glyphosate is approved for use in the European Union as an active ingredient until 15 December 2022. For renewal, the Glyphosate Renewal Group sent an application of approval to the Assessment Group on Glyphosate (AGG), the other Member States, the EFSA and the European Commission on 12 December 2019. The act formally initiates the renewal process and a full dossier of the submission has to be sent until 15 June 2020. The AGG (France, Hungary, the Netherlands and Sweden have agreed to be a part of it) will then start a comprehensive scientific evaluation taking into account progress in science and technology and experience gained since the active substance was last reviewed.

In summary, the present study indicates that glyphosate is the least toxic compound in the glyphosate-based formulations. Co-formulants are not inert, between which synergistic effects could be be greater than those with glyphosate. 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 anyone of these products, which is also an urgent task.to which the results of this study can contribute.

5. Conclusions

POE(15) containing glyphosate formulations (GBHs) are banned since 2016 in the EU because of the toxicity of POE(15). Since then, numerous GBHs are available freely with other co-formulants, which are declared as "inert compounds". In our study, most of the investigated eleven free-marketed POE(15)-free GBHs exhibited acute and chronic cytotoxicity and direct estrogenic and androgenic effects, while the pure active ingredient glyphosate acid proved to be ineffective in the applied biotests. Connection could not be found between the biological effects and the type or concentration of glyphosate salt; therefore it can be concluded that toxicity and hormonal activity are linked to the formulation. Comprehensive toxicological evaluation of the chemicals used as co-formulants in GBHs and the revision of the free availability of these formulations are pressing issues.

CRediT authorship contribution statement

Gergő Tóth: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Judit Háhn:** Conceptualization, Investigation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Júlia Radó:** Investigation. **Diána A. Szalai:** Investigation. **Balázs Kriszt:** Resources, Project administration, Funding acquisition. **Sándor Szoboszlay:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115027.

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