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Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations

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Declaration of conflict of interest

None.

Abstract

Humans are exposed to complex chemical mixtures, such as pesticides. Although the need for the assessment of health and environmental hazards deriving from the interactions between various substances found in commercial pesticide formulations is becoming increasingly recognized, the approval of pesticide products is still mostly limited to determining the toxicity of the individual ingredients ignoring the possible combined effects in mixtures. The objective of this review was to systematically review the literature of *in vitro* and *in vivo* studies that simultaneously examine the toxicity of pesticide product formulations and their declared active ingredients to compare their toxicity to human health and to the environment.

Two electronic databases were searched for studies that assessed the health effects of active pesticide ingredients and their product formulations. The literature search was performed with a combination of the following terms: “pesticide”, “formulation”, “commercial product”, “commercial pesticide” and “health”. After screening by predefined inclusion and exclusion criteria, quality and reliability assessment of eligible publications was conducted by use of the ToxRTool. Two investigators independently screened the identified publications and extracted results from eligible studies.

Our search yielded 36 toxicity studies, including 23 studies investigated herbicides, 15 examined insecticides, and 4 focused on fungicides. Twenty-four studies reported increased toxicity of the product formulations versus their active ingredients, which in most cases was attributed to the presence of adjuvants in the formulations. A significant number (n=10) of studies focused on the comparative testing of glyphosate and glyphosate-based herbicides, and six of them concluded that Roundup, the dominant product formulation of glyphosate, is more toxic than the active ingredient alone. We identified only 8 studies demonstrating reduced toxicity of product formulations in relation to the active ingredient that might be due to a potential antagonistic effect between the constituents.

The results of this review demonstrate the inadequacy of current EU testing requirements for assessing the health hazards of pesticide product formulations based mainly on the evaluation of the individual ingredients and of at least one representative use and formulation. Ignoring the possible risks deriving from the interaction between the active and other ingredients of various commercial pesticide product formulations might result in the misinterpretation of its toxicological profile. At EU level efforts are currently made to addresses this issue. In this context, we recommend that all product formulations should be fully assessed during the authorization process.

Keywords: pesticide, formulation, active ingredient, toxicity, systematic literature review

1. Introduction

The fast industrialization and development of agricultural technology over the past century have dramatically changed both the ways and the degree of human exposure to synthetic chemicals. Nowadays, one of the major challenges that our society has to face is simply to keep pace with knowing how many chemicals are produced and used in the world. This is an important starting point for identifying the hazards and estimating the risks that chemicals might pose to the environment and to human health. According to the world's largest chemical substance information centre (CAS Registry), more than 149 million unique organic and inorganic chemicals such as alloys, coordination compounds, minerals, mixtures, polymers and salts have been synthetized in the history of humanity (CAS Registry, 2019); from which at least 84,000 chemical substances, including pesticides, may possibly be in commerce (GAO, 2013). The European Commission's pesticide database currently registers 1,387 active pesticide ingredients from which 466 are permitted to use in the European Union (EU) (European Comission, 2019). The approval of active pesticide ingredients is carried out on EU level; however, the authorisation process requires that at least one representative use and formulation is assessed and deemed acceptable. The subsequent toxicological assessment and the authorisation of formulations containing an EU approved active substance are the exclusive responsibility of individual Member States (European Comission, 2018). As one active ingredient can serve as a base for many pesticide product formulations (PPFs), which can also differ in composition from one Member State to another, the number of commercially available PPFs cannot be precisely estimated but is almost certainly larger by magnitudes than the number of active substances.

The primary route of exposure to pesticides for the general population is ingestion of food products that can be contaminated with residues of, for example, insecticides, herbicides or fungicides sprayed on the crops. PPFs are not only used in agriculture but also in public applications and domestically, that provide additional routes of exposure for citizens, mainly through inhalation and direct skin contact. Moreover, all exposure pathways can affect workers, primarily pesticide handlers. It is well recognized that some pesticides can produce acute and chronic side effects in individuals when exposed, such as impairment of the central or peripheral nervous system, (neuro)endocrine effects, altered metabolic and reproductive functions, or even mutation and cancer (Mostafalou and Abdollahi, 2017).

PPFs are typically cocktails of one or more active ingredients and other substances that have no direct biocidal action, therefore labelled as "inerts", "adjuvants" or "co-formulants". These

other substances are added to the active ingredient in order to improve its dissolution, stability, absorption and pesticidal action of the active ingredient (Cox and Surgan, 2006). The main co-formulants are surfactants, including the most common non-ionic surfactants, such as ethoxylated alkylphenols, which increase the solubility of the active ingredient by forming micelles and protect it from natural degradation (Cserhati, 1995). Organic and inorganic solvents can also be used to boost the mobility of the active ingredient (Yusoff et al., 2016). The necessity of adding adjuvants depends on the physicochemical properties of the active ingredient, as well as on the state of the product formulation (suspension concentrate, solution, wettable powder, granules, etc.). The presence of adjuvants may not only promote the penetration of the active ingredient into the target organism, but also into the skin of exposed individuals. On the other hand, the supposedly "inert" adjuvants can have biological activity on their own and may be just as or even more toxic to humans than the active pesticide ingredients, or the components might have synergetic effects (Cox and Surgan, 2006). According to the US Environmental Protection Agency, it is expected that "on the order of 50% of inert ingredients would be of low or low/moderate risk" (U.S. EPA, 2002). Nonetheless, in the recent past, the exact composition of pesticide products and the identity of other ingredients in these formulations were often undisclosed because producer companies were not obligated by law to give out information on other ingredients to consumers, unless they intrinsically bore a hazardous potential to human health or to the environment. The hazards and related risks of PPFs were assessed by the toxicity of the active ingredient, largely neglecting the potential harm of other components and/or the possible combined effects of mixtures. Furthermore, formulations are not appropriately assessed since dose-dependent effects and the environmental fate of the formulants prior to reaching recipient non-target receptors are not usually taken into account. Currently, the EU limits the amount of active ingredients of pesticides allowed in food or water; however, concerns are increasing that analysing the individual ingredients and only limited number of formulations does not provide sufficient information on the potential spectrum of health effects of the widely diverse PPFs and that the interactions of ingredients should be addressed in a more systematic way to allow for effective protection of human health.

To the best of our knowledge, no systematic review has been carried out to assess whether PPFs pose greater health risk than their active ingredients. Therefore, the objective of this review was to give an overview on the literature of *in vitro* and *in vivo* studies that simultaneously examine the toxicity of PPFs and their declared active ingredients to identify the possible differences in their toxicity to human health and to the environment.

2. Materials and methods

2.1. Research question

A PECO (population, exposure, comparator, outcome) statement was developed to address and understand potential differences in the toxicity of PPFs and their declared active ingredients on human health and on the environment (Table 1).

Table 1. PECO (population, exposure, comparator, outcome) statement.

PECO element	Description
Population	Any human subjects studied prospectively without restrictions on country, race, religion, sex. Any animal model without restriction on sex, age, life stage. Any microorganism or any cell lines or *in vitro* procedures.
Exposure	Exposure to any pesticide product formulation(s) including all ranges of concentrations, duration, and routes of exposure
Comparator	A comparison group exposed to equivalent or comparable level of the active ingredient(s) of the same pesticide product formulation(s)
Outcome	Any adverse health effects or toxicity endpoints but not the intended pesticidal effect.

2.2. Identification of studies

PubMed and Scopus electronic databases were systematically searched on 08/07/2019 to identify studies from the past four decades comparatively assessing, in some way, the adverse health effects of active pesticide ingredients and their product formulations. The search was executed without any restrictions and was conducted using the following combinations of search terms: pesticide AND formulation OR commercial product OR commercial pesticide AND health. The above search terms were combined with their corresponding Medicine's Medical Subject Headings (MeSH) terms when database search was launched in PubMed. The full search string used in each database is available in the Online Supplementary Material S1. The citations of the search results were imported into a systematic review web application (Rayyan) (Ouzzani et al., 2016), and titles and abstracts of the retrieved studies were screened by two independent reviewers (K.N. and S.L.) based on predetermined inclusion and exclusion criteria. After title and abstract screening, the retrieved full-text publications of all

potentially eligible articles were independently reviewed by two reviewers (K.N. and S.L.) again, according to the same predefined inclusion and exclusion criteria. When consensus regarding the eligibility could not be reached, discrepancies were remedied by the decision of a third reviewer (B.A.).

2.3. Assessment of study eligibility

Studies meeting the following inclusion criteria were included in the review: (1) reported original, empirical research published in a peer-reviewed journal, (2) simultaneously evaluated the toxicity of pesticide formulation(s) and its/their declared active ingredient(s), (3) reported toxicity endpoints as outcome, and (4) written in English language.

The exclusion criteria were as follows: (1) non-experimental studies, (2) studies that evaluated only the pesticidal action of formulation(s) and its/their declared active ingredient(s) but not their adverse effects (3) studies with no toxicity endpoints reported, (4) studies with full-text published in a language other than English, and (5) studies with no full text available if contact to authors was unsuccessful. Review articles were excluded; however, their reference lists were manually searched for eligible articles.

2.4. Reliability and quality assessment

Reliability and quality assessment of all eligible full text articles were performed by two reviewers (K.N. and R.D.) using ToxRTool (Toxicological data Reliability Assessment Tool) which was developed by Schneider and colleagues (Schneider et al., 2009) and "provides comprehensive criteria and guidance for evaluations of the inherent quality of toxicological data, thus making the decision process of assigning reliability categories more transparent and harmonised" (European Commission, 2009). It consists of two software-based tools, one with 21 criteria to score *in vivo* and the other with 18 criteria to score *in vitro* studies. According to the scores, studies were assigned to the Klimisch categories 1, 2 or 3 that evaluate toxicological studies based on reliability criteria (Klimisch et al., 1997). According to Klimisch and colleagues, reliability is "the inherent quality of a test report or publication relating to preferably standardized methodology and the way the experimental procedures and results are described to give evidence of the clarity and plausibility of the findings." Category 1 indicates that a study is "reliable without restriction" since it was carried out according to valid and/or internationally accepted testing guidelines (preferably performed

according to Good Laboratory Practice, GLP). Category 2 indicates that a study is "reliable with restrictions" because not necessarily follows the GLP testing rules but nevertheless well documented and scientifically acceptable. Category 3 indicates that a study is "not reliable" due to substantial methodological deficiencies and insufficient documentation, but, depending on the shortcomings of the study, it may still be useful as supportive information. To determine the extent of agreement between reviewers assessing reliability, inter-rater consistency value (Cronbach α) was calculated from the scores assigned to the selected studies.

2.5. Data extraction

Two separate reviewers carried out the data extraction using two Microsoft Excel based data extraction sheets, one for *in vivo* and another for *in vitro* studies. The data extraction sheets were developed for this study and pilot tested. Data retrieved included publication details (identification, declaration on conflict of interests and funding), properties of the active compounds and formulations, methodological characteristics (concentration/dose, treatment/route of exposure, cell type/test animal, assay type), endpoint measures, response of the active compound and formulation, difference in the toxicity between the active compound and the formulation. Extracted data were descriptively analyzed using Microsoft Excel 2016.

3. Results

3.1. Identification of eligible studies

The process of the search is presented in a PRISMA flow diagram (Fig. 1). Our initial database search yielded 1094 studies, of which 37 met the inclusion and exclusion criteria after removing duplicates. No additional records were identified by hand-searching reference lists. Full texts of two studies were not available despite contacting the authors. The included studies were submitted to reliability and quality assessment. According to the Klimisch categories of ToxRTool, one study received 10 points from the maximum score of 18, therefore was excluded at this stage. The Cronbach α statistic was 0.733 for the reliability assessment, indicating acceptable internal consistency. Finally, 36 studies were included into the present systematic review.

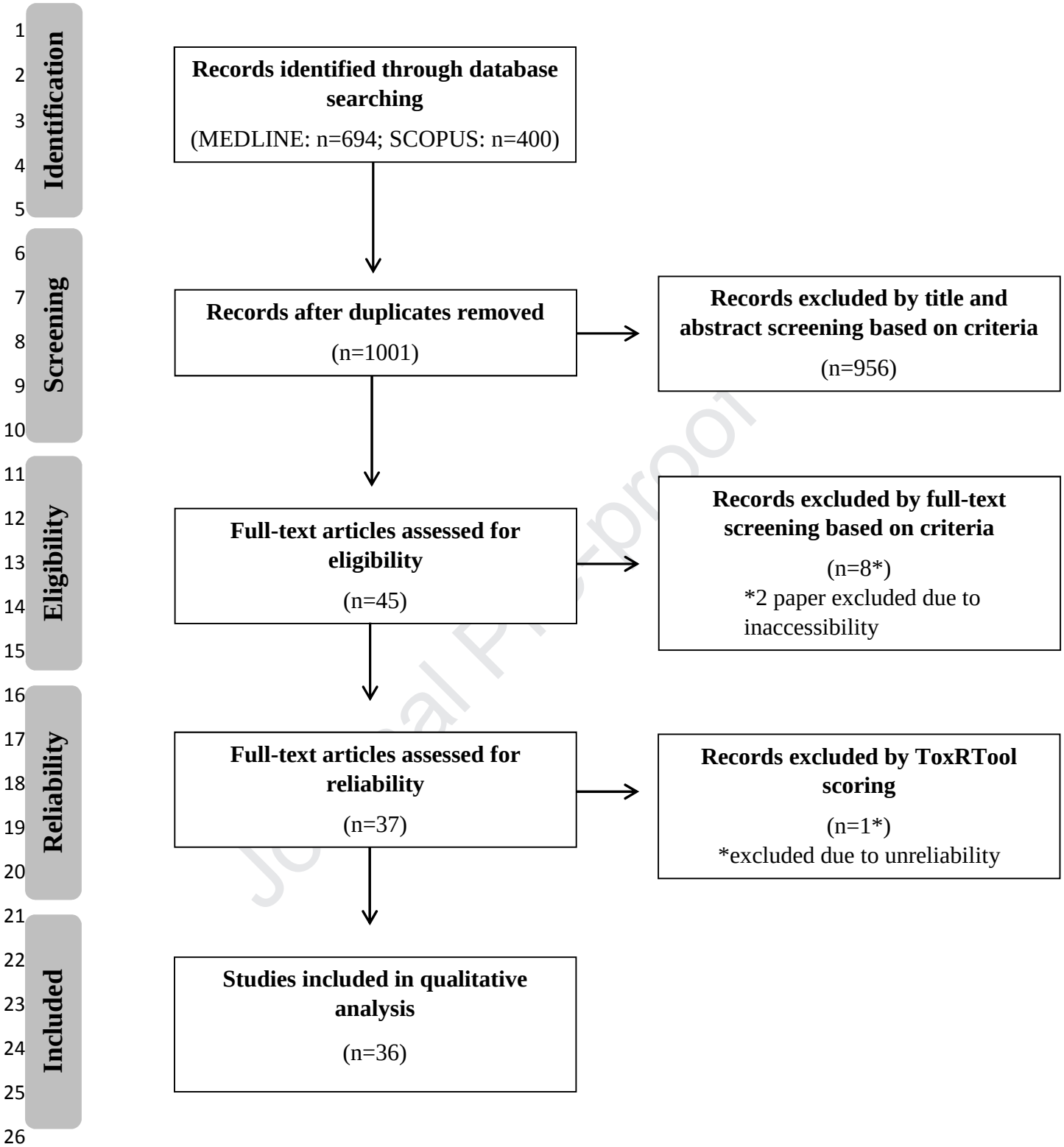

Fig. 1. PRISMA flowchart of study selection.

3.2. Summary of results of included studies

The primary findings from the included *in vivo* and *in vitro* studies are summarized by pesticide groups in Table 2-4. Eighteen studies applied *in vivo* and fifteen *in vitro* models and three studies included both *in vivo* and *in vitro* procedures. A total of twenty-three studies investigated herbicides, fifteen examined insecticides, and only four studies dealt with fungicides. Nine toxicological studies were conducted on human primary or secondary cell cultures, while the other twenty-seven studies utilized cells isolated from animals, whole animal models or bacteria, and one study also involved cells isolated from a plant (*Allium cepa*) (Grillo et al., 2014). Characteristics of the included studies are described in details by pesticide groups.

Table 2. Characteristics of the selected studies testing herbicides.

Active ingredient	Product formulation	Concentartions/ doses[a]	Duration of exposure	Cell/species	Test	Outcome description	Conclusion	Product formulation vs. active ingredient[b]	Klimisch category R_1/R_2[c,+]	Reference
In vitro										
Glyphosate	Roundup WeatherMAX, Glyfos, Roundup Classic, Kapazin, Total, Medallon Premium,	0-10,000 ppm [0-59.15 mM]	24 h	Human placental choriocarcinoma cell line (JEG3)	MTT assay, ToxiLight BioAssay, tritiated water release assay	Cytotoxicity measured as succinate dehydrogenase activity, mitochondrial respiration and adenylate kinase activity; endocrine disruption measured as aromatase activity	Product formulations containing alkyl polyglucoside and polyethoxylated tallow amine as surfactant were 15–18 times and 1200–2000 times more cytotoxic than glyphosate, respectively. All product formulations inhibited aromatase activity and disrupted mitochondrial respiration at much lower concentrations than glyphosate.	↑	1/1	(Defarge et al., 2016)
Glyphosate, isoproturon, fluroxypyr	Roundup GT+, Matin EL, Starane 200	0-10,000 ppm [0-59.15 mM] [0-48.47 mM] [0-39.21 mM]	24 h	Human embryonic kidney cell line (HEK293), human hepatoma cell line (HepG2), human placental choriocarcinoma cell line (JEG3)	MTT assay, ToxiLight BioAssay, caspase-Glo 3/7 assay	Cytotoxicity measured as mitochondrial succinate dehydrogenase activity, adenylate kinase activity and caspase 3/7 activity	All product formulations were cytotoxic and far more toxic than their active ingredients, except for isoproturon and its formulated product Matin which were both not soluble over 100 ppm [0.48 mM]. Roundup GT+ was found to be 125 times more toxic than glyphosate. Roundup was by far the most toxic among the herbicides and insecticides tested in this experimental system.	↑	2/1	(Mesnage et al., 2014)
Glyphosate	Roundup R400, Roundup R450	0-10,000 ppm [0-59.15 mM]	24 and 48 h	*G. candidum* *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *cremoris*	Turbidimetry, colony counting and observation	Growth inhibition, minimal inhibitory concentration (MIC), minimal microbicide concentration (MMC), colony description	Roundup had an inhibitory effect on microbial growth and a microbicide effect at lower concentrations than those recommended in agriculture. Glyphosate at these levels had no significant effect on the studied microorganisms.	↑	3/2	(Clair et al., 2012)
Glyphosate	Roundup	0-15 mM	5 min	Rat liver mitochondria	Mitochondrial respiratory activity measurements,	Succinate-dependent respiratory indexes, oxygen consumption,	Roundup stimulated succinate-supported respiration twice, with simultaneous collapse of transmembrane electrical potential,	↑	2/1	(Peixoto, 2005)

					membrane potential measurements, enzymatic activity measurements, measurement of mitochondrial swelling	mitochondrial membrane potential, ATPase and ATPsynthase, enzymatic activities, mitochondrial swelling, mitochondrial permeability transition	while glyphosate used in the same concentrations did not induce any significant effect. Glyphosate was not capable of crossing the lipid membrane. Under these experimental conditions, glyphosate did not have any significant effect on the mitochondrial state 4, on state 3, and on uncoupled respiration whereas Roundup inhibited the state 3 and the uncoupled respiration.			
Glyphosate	Roundup	0-2 % in cell culture medium [0-42.58 mM]	1, 18, 24 and 48 h	Human placental choriocarcinoma cell line (JEG3)	MTT assay, radioimmunoassay, tritiated water release, chromatographic separation, measurement of reductase activity	Cell viability, aromatase activity, 1/aromatase activity, aromatase mRNA level measurement, reductase activity	Roundup reduced JEG3 cell viability at least twice more efficiently than glyphosate. The dilution of glyphosate in the Roundup formulation may multiply its endocrine effect.	↑	2/1	(Richard et al., 2005)
Paraquat	Chitosan/tripolyphos-phate nanoparticles loaded with paraquat	0.0048 to 0.12 (0.38 for Allium cepa) mg/ml [18.66 to 466.64 µM (1.48 mM for *Allium cepa*)]	24 h	Chinese Hamster Ovary cells (CHO), root cells of *Allium cepa*	MTT assay, *Allium cepa* chromosome aberration assay	Cell viability (%), chromosome aberrations in *Allium cepa* root cells	Cytotoxicity and genotoxicity assays showed that the nano-encapsulated herbicide was less toxic than the pure compound. The MTT test showed that association of the herbicide with the particles reduced its cytotoxicity towards CHO cells, whereas *Allium cepa* chromosome aberration assays demonstrated that the particle-bound paraquat caused less chromosome damage, compared to the free herbicide.	↓	2/2	(Grillo et al., 2014)
Paraquat	a supramolecular formulation of paraquat@cucurbit[7]uril (PQ@CB[7])	0.1, 0.2, and 0.5 mM paraquat; 0, 0.2, 0.5, 1, and 2 mM PQ@CB[7]	6, 12 and 24 h	Murine macrophage RAW264.7 cell line	Cell Counting Kit-8 (CCK-8), annexin V-FITC/PI staining, 2′,7′-Dichloro-dihydrofluorescein diacetate (DCFH-DA)	Cell viability (%), apoptosis (%), reactive oxygen species (ROS) generation	Supramolecular paraquat encapsulation by the artificial receptor CB[7] reduced intracellular generation of ROS, leading to alleviation of apoptosis and enhanced cellular viability.	↓	1/2	(Zhang et al., 2019)

					assay				
Atrazine	Gesaprim	0.047, 0.47 and 4.7 µg/ml [0.22, 2.18 and 21.79 µM]	0.5, 1, 3, 5 and 8 h	Isolated human lymphocytes	Trypan blue exclusion assay, comet assay, DNA diffusion assay	DNA damage, induction of apoptosis and necrosis	Atrazine did not appear to be genotoxic or to be capable of inducing apoptosis or necrosis. Unlike atrazine, Gesaprime increased DNA damage in lymphocytes.	↑	1/1	(Zeljezic et al., 2006)
Alachlor	Lasso	1.0, 0.1 and 0.01 mM	4,5 and 7 h, as well as 10 days depending on the tests	Isolated human mononuclear cells	Lymphocyte transformation assay, antibody synthesis assay, particle concentration fluorescence immunoassay, natural killer cell assay, lymphokine activated killer cell assay, mixed lymphocyte culture assay	Human immune function	Lasso and alachlor had no effect on the non-specific or antigen specific proliferation of T cells. The same compounds had no effect on the proliferation and antibody synthesis by B cells. The function of killer cells was not affected by exposure to the chemicals.	-	1/1[+]	(Flaherty et al., 1992)
2,4-dichlorophenoxyacetic acid (2,4-D)	Vesakontuho Tasku	0-0.5 mM of active ingredient 0-1.5 mM of formulation	24 h	Isolated human lymphocytes	Chromosome aberration assay	Chromatide type and chromosome type aberrations	Pure 2,4-dichlorophenoxyacetic acid did not increase the number of aberrations, whereas the commercial 2,4-D formulation (0.125, 0.250, 0.500, 1.000 and 1.250 mM, with respect to phenoxyacetic acid concentration) significantly increased the number of chromosome aberrations in vitro (with exogenous metabolic activation).	↑	1/1	(Mustonen et al., 1986)
2,4-dichlorophenoxyacetic acid (2,4-D)	Spurge & Oxalis Killer	0, 0.005 and 0.3 mM	48 h	Isolated human lymphocytes	Micronucleus assay	Micronucleus frequency, replicative index (RI), mitotoc index (MI)	The overall genotoxic effect of both pure and commercial 2,4-D as measured by the micronucleus assay was minimal. Lymphocyte RI was more affected by commercial 2,4-D than with an equal concentration of the pure 2,4-D suggesting that other ingredients present in the	- ↑	1/1	(Holland et al., 2002)

							commercial pesticide may be responsible or may enhance the effect of 2,4-D.			
In vivo										
Glyphosate	Spasor	*Pseudokirchneriella subcapitata*: 0-237 mg/l [0-1.40 mM] *Daphnia magna*: 0-up to 2000 mg/l [0-2.09 mM] *Eisenia andrei*: 0-165 mg/kg	96 h, 48 h, 48h	Microalga (*Pseudokirchneriella subcapitata*), *Daphnia magna*, Earthworms (*Eisenia andrei*)	Algal growth assay (OECD 2006), Daphnia immobilisation assays (OECD 2004), Earthworm avoidance assays	Growth inhibition for green alga, immobilisation for *Daphnia magna*, avoidance behaviour for earthworm *Eisenia Andrei*	*D. magna* was found fairly insensitive to glyphosate and Spasor. Contrarily, toxicity of glyphosate/Spasor to aquatic organisms (microalgae) clearly demonstrated that formulation ingredients enhanced herbicide toxicity.	↑	1/2	(Pereira et al., 2009)
Glyphosate	Roundup Ultra	0-116 µg/ml [0-686.10 µM]	1 and 3 days	European eel (*Anguilla anguilla L.*)	Comet assay	DNA damage	Roundup revealed to be less genotoxic than the active ingredient or the surfactant alone (with the exception of the higher concentrations after 3 days). Fish exposed to Roundup always showed a level of damage lower than expected based on the sum of the effects of the separate components, suggesting an antagonistic interaction between glyphosate and polyethoxylated tallowamine.	↓	1/1	(Guilherme et al., 2012)
Glyphosate	Roundup	1.75 mg/kg bw/day diluted in water (equivalent for formulation)	6 and 13 weeks water ad libitum	Sprague Dawley rats	Modified One-Generation study (based on U.S. National Toxicology Program)	Evaluation of *in vivo* parameters and the determination of glyphosate and its major metabolite AMPA in urine	Both glyphosate and Roundup exposure led to comparable urinary concentrations of glyphosate and aminomethylphosphonic acid (AMPA) with an increasing pattern of glyphosate excreted in urine in relation to the duration of treatment, indicating the systemic bioavailability of the active substance and a possible mechanism of bioaccumulation.	-	1/2	(Panzacchi et al., 2018)

Glyphosate	Glyfonova® 450 Plus, Roundup® Garden	2.5 mg/kg/day (GLY5) and 25 mg/kg/day (GLY50) of glyphosate, 25 mg/kg/day of Glyfonova® (NOVA)	2-weeks (oral gavage)	Sprague Dawley rats	Effects on the intestinal microbial composition, activity and host response during a short-term exposure trial	Effects on intestinal microbial communities	No physiological abnormalities were observed in organs from the rats dosed with glyphosate. However, slightly increased serum levels of the acute phase protein haptoglobin were found in rats treated with Glyfonova®. This suggests that this reaction could be caused by additives in the formulation, which warrants further investigation. no effect of Glyfonova® on levels of serum IL-6, which is known to induce the acute phase protein.	↑	1/1	(Nielsen et al., 2018)
Diuron	Nortox	*D.furcatus and Allonais inaequalis:* 2 to 64 mg/l for diuron and 1 to 32 mg/l for Nortox® 500 *Strispinosa trispinosa*: 8 to 32 mg/l for the active ingredient diuron and 1 to 32 mg/l for Nortox® .	96-h exposure for *A. inaequalis* and *D. furcatus*, 48 h for *S.trispinosa*	*A. inaequalis*, *D. furcatus*, *S. trispinosa*	Acute toxicity test	Mortality/immobility (EC 50)	The toxicity of product formulations was significantly greater than their corresponding active ingredients in all cases.	↑	1/1	(Rocha et al., 2018)
Trifluralin	Treflan 4D, Prowl 400EC,	Treflan 4D and Prowl 400EC: 0-8 mg/l, Trifluralin 0-10.125 mg/l	96 h (acute)	Green frogs	Acute and chronic toxicity test	96-h mortality, LC50	Treflan 4D, Prowl 400EC were more toxic at lower concentrations than the active ingredient alone.	↑	1/1	(Weir et al., 2012)
2,4-dichlorophenoxyacetic acid (2,4-D)	Vesakontuho Tasku	0.03-0.04 mg/m^3 in workplace air	24 h	Occupationally exposed human subjects and unexposed controls	Chromosomal aberrations test	Chromatid type and chromosome type aberrations	*In vivo* study did not reveal any significant increases in chromosomal aberrations in lymphocytes of humans occupationally exposed to phenoxy acid herbicides formulation.	-	1/1	(Mustonen et al., 1986)
2,4-dichlorophenoxyacetic acid	2,4-D LV4	0-500 µl/ml of water	10 days	Lady beetles	Acute toxicity test	Larval mortality, development rates and mobility	The most toxic was the 2,4-D LV4 formulated product, with a survival rate reduced by 80% for Lady	↑	2/1$^+$	(Freydier and Lundgren, 2016)

(2,4-D) Dicamba	Dicamba DMA						beetles larvae. The dicamba active ingredient also significantly reduced the longevity and the survival of larvae, but not Dicamba DMA.	↓	
Atrazine S-metolachlor	Atrazine + technical grade S-metolachlor Bicep II Magnum	0.01 ppb and 10 ppb	Form 2 days posthatching until complete tail reabsorption	Leopard frogs	Larval growth, metamorphosis, gonadal differentiation and immunocompetence	Size and time to metamorphosis, histological analysis of gonads and thymus	The product formulation (Bicep II Magnum) appeared less toxic than the pure mixture of atrazine and S-metolachlor. The surfactant used in this mixture reduced the toxic effects of the two pesticide active ingredients.	↓	2/1[+] (Hayes et al., 2006)
Propanil	Stam Novel Flo 480	*Pseudokirchneriella subcapitata*: 20.7-156 mg/l, *Daphnia magna*: 1.19-9.49 mg/l, *Eisenia andrei*: 2.2-16.4 mg/kg	96 h, 48 h, 48 h	Microalga; *Daphnia magna*; Earthworms	Algal growth assay, Daphnia immobilization assays, Earthworm avoidance assays	Growth inhibition for green alga; immobilization for *Daphnia magna*; avoidance behaviour for earthworm	Significant difference between the toxic effect of the active ingredient and the commercial product formulation was not observed.	-	1/2 (Pereira et al., 2009)
Azadirachtin	Neemix Bioneem	0, 0.0156, 0.0313, 0.0625, 0.125, 0.25, and 0.5 mg/ml	24, 48, 72, and 96 h depending on cell types	*Daphnia pulex*	Water flea toxicity test	Daphnia mortality rate (LC50)	Azadirachtin-based pesticides and pure azadirachtin preparations were tested at equivalent azadirachtin concentrations, pure azadirachtin was found to be less toxic to *D. pulex*.	↑	2/1 (Goktepe and Plhak, 2003)
Paraquat	a supramolecular formulation of paraquat@cucurbit[7]uril (PQ@CB[7])	Paraquat: 300 mg/kg dose; 1:2 molar ratio, paraquat and CB[7] of 300 mg/kg and 2.71 g/kg, respectively	8 days	Wild-type zebrafish	Behaviour monitoring	Survival, signs of sickness	The supramolecular formulation exhibited significantly reduced hepatotoxicity and increased survival rate, in comparison with those of the fish exposed to free PQ.		1/2 (Zhang et al., 2019)
				Mice (Balb/c, male)	Survival rate, histopathological analysis, haematological and hepatic parameters	Survival, tissues damage, haematotoxicity and hepatotoxicity	Survival rate was dramatically improved when the mice were fed with the supramolecular formulation of PQ (PQ@CB[7] 1:1 and 1:2) at a supralethal dose (300 mg/kg) or ultrasupralethal dose (600 mg/kg) of PQ.	↓	

Terbuthylazine	Radazin TZ-50	0.0035 mg/kg bw/day	14 days	Male Swiss albino mice	Comet assay	DNA damage	Exposure to both of the tested compounds resulted in significantly increased mean tail lengths and tail intensities in leukocytes, bone marrow cells, and liver cells compared to the values measured in respective control groups. In kidney cells, only exposure to the formulated product Radazin TZ-50 caused a significant increase of mean tail lengths and tail intensities compared to the values measured in control mice.	-/↑	1/1	(Zeljezic et al., 2018)
Tribenuron-methyl	Granstar	25, 50 and 100 mg/kg bw	Single oral dose or 10 ripetitive oral dose	*Rattus norvegicus*	Chromosomal abnormalities; Mitotic index determination; Micronucleus assay;	Cytogenic effects; Cell proliferation; Percentage frequency in micronucleus	The partial differences of the genotoxic effects obtained with pure and commercial tribenuron-methyl indicate that commercial formulations may contain additional hazardous compounds.	↑	3/1	(Mamdouh et al., 2012)
Pendimethalin	Prowl 400	Pendimethalin: 500 and 800 ng/mL Prowl 400: 500 ng/mL	28 days	Rainbow trout (*Oncorhynchus mykiss*)	*In vivo* chronic contamination effects	Bioconcentration in flesh; Physiological status parameters (Weight/length %, weight of liver/weight of fish%) Haematology and immune parameters	Chronic exposure to pendimethalin alone or with adjuvants affected the sanitary status as well as to immune responses in trout. Prowl 400® was evaluated more immunotoxic than Pendimethalin alone.	↑	1/1	(Danion et al., 2012)

[a]equivalent concentration/dose of active ingredient alone and in formulation unless otherwise stated

[b](↑) higher, (↓) lower or no change (-) in the toxicity of the formulation compared to the active ingredient

[c]Klimisch categories indicating the reliability of the study classified by the first (R_1) and the second (R_2) reviewer

[+]existence of potential conflicts of interests

Table 3. Characteristics of the selected studies testing insecticides.

Active ingredient	Product formulation	Concentrations/ doses[a]	Duration of exposure	Cell/species	Test	Outcome description	Conclusion	Product formulation vs. active ingredient[b]	Klimisch category R_1/R_2[c,+]	Reference
In vitro										
Avermectin, hexaflumuron, chlorfluazuron, chlorpyrifos, tebufenozide	No ready-to-use formulations; surfactants (Tween 80 and PEG6000) are manually added to the solution of active ingredients before application	20 µM of active ingredient 0, 4, 8, 16 µM of surfactants	24 and 72 h	Insect cell line from *Trichopulsi ni* (Tn5B1-4), human hepatoma cell line (HepG2)	MTT assay	Cell viability (%)	Co-incubation of avermectin with nontoxic concentrations of Tween 80 and PEG6000 powerfully counteracted the cytotoxicity. The cytotoxicity of chlorfluazuron against Tn5B1-4 cells was enhanced by Tween 80 whereas compressed by PEG6000. Co-incubation with Tween 80 increased the cytotoxicity of chlorpyrifos or tebufenozide on HepG2 cells.	↓	2/1	(Li et al., 2015)
Pirimicarb, imidacloprid, acetamiprid	Pirimor G, Confidor, Polysect Ultra	0-10,000 ppm [0-41.97 mM] [0-39.11 mM] [0-44.91 mM)	24 h	Human embryonic kidney cell line (HEK293), human hepatoma cell line (HepG2), human placental choriocarcinoma cell line (JEG3)	MTT assay, ToxiLight BioAssay, caspase-Glo 3/7 assay	Cytotoxicity measured as mitochondrial succinate dehydrogenase activity, adenylate kinase activity and caspase 3/7 activity	All product formulations were cytotoxic and far more toxic than their active ingredients.	↑	2/1	(Mesnage et al., 2014)
Pirimicarb	Aficida	0-300 µg/mL [0-1.26 mM]	24 h	Chinese Hamster Ovary cells (CHO)	Cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay, neutral red and MTT assays, flow cytometry using annexin V-FITC/PI double staining	Micronucleus (MNs) induction, nuclear division index (NDI), cell viability (%), succinic dehydrogenase activity (%), apoptosis, necrosis (%)	The capacity of pirimicarb and Aficida to induce MNs was found to be equivalent for all concentration assayed. The pure active ingredient more actively induced cytotoxic effects in CHO-K1 cells than its commercial formulation.	↓	1/1	(Soloneski et al., 2015)
Amitraz	Azadieno	1.25, 2.5 and 3.75 µg/mL [4.26, 8.52 and 12.78 µM]	16 h	Chinese Hamster Ovary cells (CHO)	Comet assay, Annexin V/PI staining assay	DNA damage, induction of apoptosis	The product formulation significantly increased the parameters of DNA damage compared to the control at lower concentration than the active	↑	2/1	(Padula et al., 2012)

							ingredient. The commercial product formulation was more effective at inducing genotoxicity and apoptosis than amitraz.			
Azadirachtin	Neemix, Bioneem	0, 0.01, 0.1, 1, 10, and 100 µg/ml [0, 0.014, 0.14, 1.38, 13.87 and 138.75 µM]	24, 48, 72, and 96 h depending on cell types	Murine hybridoma cell line, isolated oyster heart cells	Trypan blue exclusion assay, MTT assay, MTS/PMS assay	Cytotoxicity	The active ingredient alone did not have any measurable harmful effects on hybridoma and oyster cells. Neemix and Bioneem, but not azadirachtin, were toxic to both hybridoma and oyster cells in a dose-response manner.	↑	2/1	(Goktepe and Plhak, 2003)
Chlorpyrifos	Rid-a-bug	0-100 µg/ml [0-285.23 µM]	5 days	Rat isolated midbrain cells	Neutral red staining, hemtoxylin staining	Cytotoxicity (%), cell differentiation	Rid-a-bug, a product formulation of chlorpyrifos, was less cytotoxic and its inhibitory concentration (IC50) was lower than the active ingredient solved in methanol, ethanol and xylene.	↓	3/2	(Cosenza and Bidanset, 1995)
In vivo										
Carbofuran	Furadan 350 SC	*Dero furcatus*: 62.5 to 4000 µg/l *Allonais inaequalis*: 62.5 to 4000 µg/l for carbofuran and 500 to 32,000 µg/l for Furadan 350 SC; *Strispinosa trispinosa*: 15.6 to 250 µg/l for carbofuran and 15.6 to 250 µg/l for Furadan 350 SC	96-h exposure for *A. inaequalis* and *D. furcatus*, 48 h for *S.trispinosa*	*A Inaequalis*, *D. Furcatus*, *S. Trispinosa*	Acute toxicity test	mortality/immobility (EC 50)	The toxicity of product formulations was significantly greater than that of the active ingredient except for the test to the ostracod *S. trispinosa*. In this case, the active ingredient carbofuran was more toxic than its product formulation.	↑ ↑ ↓	1/1	(Rocha et al., 2018)
Methomyl	Lannate	*Pseudokirchneriella subcapitata*: 62.5-350 mg/l *Daphnia magna*: 13-45 mg/l *Eisenia andrei*:	96h, 48h, 48h	Microalga *Daphnia magna*; Earthworms	Algal growth assay, Daphnia immobilisation assays, Earthworm avoidance	Growth inhibition for green alga, immobilisation for *Daphnia magna*, avoidance behaviour for	The toxicity of the product formulation was lower to microalgae than that of the active ingredient. The same level of toxic effect was	↓ -	1/2	(Pereira et al., 2009)

		1.36-23 mg/kg			assays	earthworm	found to *D. magna* when exposed to Lannate and methomyl.			
							Earthworms significantly avoided the soil contaminated with the formulation at lower concentrations than with the active ingredient.	↑		
Fipronil	Adonis 3UL	8.7, 12.5 and 18.3 ml solution (0.3% fipronil and 12% diacetone alcohol)/kg bw	single oral dose 48 h	Birds (adult male zebra finches)	Acute oral toxicity test	*e*LD50	Adonis 3UL, as the product formulation of fipronil, had greater toxic effect than the active ingredient alone.	↑	1/2	(Kitulagodage et al., 2008)
Resmethrin	Scourge	Adult toxicity test (24 h): 1.56 - 25.0 µg/l of resmethrin and 6.25 - 100 µg/l of Scourge, Adult toxicity test (96 h): 0.125 - 2.0 µg/l of resmethrin and 0.2 - 16.2 µg/l of Scourge, Larval toxicity test (24 h): 0.625 - 10.0 µg/l of resmethrin and 5.1 - 81.0 µg/l of Scourge, Adult toxicity test (96 h): 0.125 - 2.0 µg/l of resmethrin and 0.2 – 16.3 µg/l of Scourge	24 h and 96 h	Grass shrimp (*Palaemonetes pugio*)	Adult shrimp toxicity tests, Larval shrimp toxicity tests	LC50	When the Scourge formulation (18% resmethrin + 54% PBO) is adjusted for resmethrin, this synergized resmethrin is seen to be more toxic to adult and larvae grass shrimp than the nonsynergized formulation except for the larval sediment exposure.	↑ ↑ - ↓	1/1	(Key et al., 2005)
Guthion	Guthion 2S	<0.01 (control) - 3.60 ± 0.35 mg/l	10 days	Frog (*Pseudacris regilla*)	Survival and growth tests	Survival, total length, salamander snout-to-vent length (SVL), and total wet	Guthion 2S was significantly more toxic than the active ingredient.	↑	1/1	(Nebeker et al., 1998)

						weight				
Deltamethrin	Decis	5 or 10 mg/kg	7 days	Male Wistar rats	Urinary and faecal mutagenicity assays	Formation of mutagenic metabolites by analyzing urinary and faecal mutagenicity	The results showed some differences in data obtained from pure and commercial deltamethrin experiments and confirmed that product formulations may contain additional hazardous compounds.	↑	1/1	(Moretti et al., 1997)
Cypermethrin Deltamethrin	Sparkle (25% active ingredient) K-Othrin (2.5% active ingredient)	Cypermethrin: 25 mg/kg bwt Deltamethrine: 1.7 mg/kg bwt	90 days	Male Wistar rats	Body and organ weight, Red and white blood cells, haematocrit value, Serum ALT and AST, total protein and creatinine	Decreasing/increasing body or organ weight, haematotoxicity, liver and kidney dysfunction	Formulated and technical insecticides induce biochemical and haematological changes in rats. Presence of other hazardous compounds in formulations may increase their toxicity.	↑	1/1	(Abbassy and Mossa, 2012)
Cypermethrin Cyphenothrin Deltamethrin Permethrin	Active ingredient + piperonyl butoxide (PBO)	Pyrethroids: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 150, and 200 ppm; PBO: 1, 5, 25, and 50 ppm	Up to 10 days	*Drosophila melanogaster*	Determination of LC_{25} and LC_{50}; Wings somatic mutation and recombination test (SMART assay)	Wings were scored for small (1-2 cells) single spots, large (>2 cells) single spots, and twin spots	Synthetic pyrethroids (cypermethrin, cyphenothrin, deltamethrin and permethrin) alone and their combinations with PBO in *D. melanogaster* measured by the SMART assay were not genotoxic.	-	1/2	(Demir et al., 2014)
Malathion Carbaryl	Ortho Ortho formulation grade carbaryl/malathion in combination at 1/1	0, 1, 10, and 100 mg/kg bw for individual active ingredients and 50/50 mg/kg bw for carbaryl/malathion combination	3 months	Sprague-Dawley rats	Maternal toxicity test	Teratogenic potential	When the carbaryl and malathion used together in a mixture, there may be increases in hazards producing maternal toxicity.	↑	2/2	(Lechner and Abdel-Rahman, 1984)

[a]equivalent concentration/dose of active ingredient alone and in formulation unless otherwise stated

[b](↑) higher, (↓) lower or no change (-) in the toxicity of the formulation compared to the active ingredient

[c]Klimisch categories indicating the reliability of the study classified by the first (R_1) and the second (R_2) reviewer

[+]existence of potential conflicts of interests

1 **Table 4.** Characteristics of the selected studies testing fungicides.

Active ingredient	Product formulation	Concentrations/ doses[a]	Duration of exposure	Cell/species	Test	Outcome description	Conclusion	Product formulation vs. active ingredient[b]	Klimisch category R_1/R_2[c,+]	Reference
In vitro										
Tebuconazole, epoxiconazole, prochloraz	Maronee, Opus, Eyetak	0-32.49 mM 0-30.33 mM 0-26.55 mM (0-10,000 ppm)	24 h	Human embryonic kidney cell line (HEK293), human hepatoma cell line (HepG2), human placental choriocarcinoma cell line (JEG3)	MTT assay, ToxiLight BioAssay, caspase-Glo 3/7 assay	Cytotoxicity measured as mitochondrial succinate dehydrogenase activity, adenylate kinase activity and caspase 3/7 activity	All product formulations were cytotoxic and far more toxic than their active ingredients.	↑	2/1	(Mesnage et al., 2014)
Zineb	Azzurro	0-100 µg/ml [0-362.58 µM]	80 min and 24 h depending on the tests	Chinese Hamster Ovary cells (CHO)	Sister chromatid exchange (SCE) assay, single cell gel electrophoresis (SCGE) assay (comet assay)	Sister chromatid exchange, cell-cycle kinetics, mitotic index	Statistically significant positive relationship was found between zineb at concentration of 25.0–100.00µg/ml and DNA damage levels. In Azzurro-treated cells, a significant increase in the frequency of damaged cells over controls was observed only at 100 µg/ml. At lower doses, no DNA damage was detected.	↓	2/1	(Soloneski et al., 2002)
Zineb	Azzurro	0-100 µg/ml [0-362.58 µM]	72 h	Human isolated mononuclear leukocytes	MAC (morphology, antibody, chromosomes) cytogenetic technique, micronucleus test	Micronucleus frequency, proportions (%) of various lymphocyte subsets among all interphase cells, proportions (%) of various lymphocyte subsets among all mitotic cells	Zineb and Azzurro cytotoxicity were observed at doses higher than 50 µg/ml. No significant differences were noted in the frequency of micronuclei induced by zineb and Azzurro in any of the lymphocyte subsets.	-	2/1	(Soloneski et al., 2001)
Zineb	Azzurro	0-50 µg/ml [0-181.29 µM]	24 h	Chinese Hamster Ovary cells (CHO)	Cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay, neutral red and MTT assays, flow cytometry using annexin V-FITC/PI	Micronucleus (MNs) induction, nuclear division index (NDI), cell viability (%), succinic dehydrogenase activity (%),	Zineb and Azzurro increased the frequency of MNs when cells were exposed within the 1–5 µg/ml and 1–10 µg/ml concentration ranges to zineb and Azzurro, respectively. The pure active ingredient more actively induced cytotoxic effects in CHO-K1 cells than its commercial formulation.	↓	1/1	(Soloneski et al., 2015)

double staining apoptosis, necrosis (%)

[a]equivalent concentration/dose of active ingredient alone and in formulation unless otherwise stated

[b](↑) higher, (↓) lower or no change (-) in the toxicity of the formulation compared to the active ingredient

[c]Klimisch categories indicating the reliability of the study classified by the first (R_1) and the second (R_2) reviewer

[+]existence of potential conflicts of interests

3.2.1 Herbicides

A majority of the identified publications (n=10) focused on the comparative testing of glyphosate and glyphosate-based herbicides (GBHs), the pesticides applied nowadays in the largest amount worldwide. Six studies concluded that Roundup, the dominant product formulation of glyphosate, is more toxic than the active ingredient alone (Clair et al., 2012; Defarge et al., 2016; Mesnage et al., 2014; Nielsen et al., 2018; Peixoto, 2005; Richard et al., 2005). One of the studies using secondary human cell cultures demonstrated that eight PPFs out of nine were up to one thousand times more cytotoxic than their stated active ingredients. Roundup was by far the most cytotoxic among the herbicides and insecticides tested in this experimental system (Mesnage et al., 2014). Besides, Roundup inhibited the growth of certain food microorganisms (Clair et al., 2012) and bioenergetics functions of isolated rat liver mitochondria (Peixoto, 2005) more efficiently than its active ingredient. The same product formulation and some other GBHs examined by Richard et al. and Defarge et al., respectively, decreased cell viability and aromatase activity, as an indication of endocrine disruption, of human placental choriocarcinoma cells (JEG3) at significantly lower concentrations than glyphosate alone which was presumably due to the presence of co-formulants in the herbicide products (Defarge et al., 2016; Richard et al., 2005). Defarge and colleagues confirmed the latter assumption by testing the toxicity of individual co-formulants of various GBHs and found that alkyl polyglucoside (APG) and polyethoxylated tallowamine (POEA) are more cytotoxic than the formulation or the active compound itself, and act as endocrine-disrupting chemicals at significantly lower levels than glyphosate alone.

A preliminary study exposing rats to glyphosate and Roundup for 6 and 13 weeks did not find any differences in urinary levels of glyphosate or the metabolite of glyphosate (aminomethylphosphonic acid, AMPA) but highlighted a systemic bioavailability of the active ingredient (Panzacchi et al., 2018). Guilherme and colleagues confirmed the genotoxicity of Roundup (at the highest concentration and both exposure times) in terms of non-specific DNA damage, and also demonstrated, in European eel, the genotoxic potential of glyphosate and POEA individually (Guilherme et al., 2012). Even so, the overall genotoxicity of Roundup in fish was lower than of the active ingredient or the POEA surfactant alone, with the exception of the higher concentrations after 3 days (Guilherme et al., 2012). Some other GBHs, such as Spasor and Glyfonova, have also showed increased toxicity in aquatic organisms and rats as compared to glyphosate alone (Nielsen et al., 2018; Pereira et al., 2009).

Two herbicides (isoproturon and fluroxypyr) and their product formulations (Matin EL and Starane 200) were also tested by Mesnage and colleagues in human secondary cell lines (Mesnage et al., 2014). Starane 200 was found to be more cytotoxic than fluroxypyr; however, it was not observed for isoproturon and its formulated pesticide Matin which can be due to the fact that Matin EL does not have any declared adjuvant.

Gesaprim® is one of the product formulations of atrazine, also a very popular herbicidal substance worldwide. Zeljezic and colleagues utilized comet and DNA diffusion assays to examine DNA damage as well as the induction of apoptosis and necrosis in isolated human lymphocytes as a result of atrazine and Gesaprim® exposure. By comparing the assay endpoints, the product formulation showed to have significant cytogenetic effect whereas atrazine alone had neither effect on DNA nor was able to induce apoptosis or necrosis of human lymphocytes (Zeljezic et al., 2006). Contrary to this study's observations, using an *in vivo* experimental system Hayes and colleagues concluded that a commercial mixture of atrazine and S-metolachlor containing surfactant as other ingredient (Bicep II Magnum) appears to be more benign on the size and time needed for *Rana pipiens* larvae to metamorphose than the pure mixture of atrazine and S-metolachlor. It suggests that the surfactant used in the product formulation reduces the effect of the two active ingredients (Hayes et al., 2006).

The toxicity of the herbicide terbuthylazine was compared with its formulation Radazin TZ-50, by Zeljezic and colleagues. The comet assay performed on mice cells revealed that, only for kidney cells, exposure to the formulated product Radazin TZ-50 caused a significant increase of mean tail lengths and tail intensities compared to the values measured in control mice (Zeljezic et al., 2018).

An experimental formulation of paraquat as an alternate weed control product was developed by Grillo and colleagues in a way that the herbicide was encapsulated into nanoparticles composed of chitosan and sodium tripolyphosphate by ionic gelification technique (Grillo et al., 2014). The cytotoxicity and genotoxicity of the free and encapsulated herbicide was then comparatively evaluated in Chinese hamster ovary (CHO) cells and isolated root cells of *Allium cepa in vitro*, respectively. The nano-encapsulated herbicide reduced its cytotoxicity towards CHO cells, while *A.cepa* assays demonstrated that the particle-bound paraquat caused less chromosome damage, compared to the free herbicide. Zhang and colleagues also prepared a novel supramolecular formulation of paraquat@cucurbit[7]uril (PQ@CB[7]) as an eco- and user-friendly herbicide by mixing paraquat with equivalent amount of an artificial receptor cucurbit[7]uril in water (Zhang et al., 2019). The resulting product maintained the

effective herbicidal activity of paraquat whilst by reducing its cellular uptake into murine cells, the product formulation decreased intracellular generation of ROS, leading to alleviation of apoptosis and enhanced cellular viability.

Flaherty and colleagues compared the immunotoxicity of the product formulation Lasso® with its active ingredient alachlor and found that neither the active ingredient nor the product formulation had any effect on human immune cells (Flaherty et al., 1992).

2,4-Dichlorophenoxyacetic acid (commonly known as 2,4-D) and its product formulation Vesakontuho Tasku® were analysed by Mustonen and colleagues with the aim of investigating and comparing their effects on the induction of chromosome aberrations in human peripheral lymphocyte cultures *in vitro* and in lymphocytes of exposed workers *in vivo*. The active ingredient 2,4-D did not increase the number of aberrations, while the product formulation significantly elevated the number of chromosome aberrations *in vitro.* The findings of the *in vivo* tests showed that exposure to the product formulation does not contribute to the induction of chromosomal aberrations in lymphocytes from humans occupationally exposed (Mustonen et al., 1986). Spurge & Oxalis Killer, another formulation of 2,4-D, enhanced the proliferation of human lymphocytes *in vitro* more than equal concentration of the pure 2,4-D suggesting that other ingredients present in the commercial pesticide may be responsible or may enhance the effect of 2,4-D (Holland et al., 2002). In a more recent study, 2,4-D and dicamba as well as their product formulations have been tested for larval mortality and development rates on *Coleomegilla maculata* larvae and found that 2,4-D formulation was the most toxic, with a reduced survival rate by 80%. Whereas for dicamba, the active ingredient was more toxic than the product formulation by significantly reducing longevity and survival of larvae (Freydier and Lundgren, 2016).

The assessment of short-term exposure to active ingredient of a contact herbicide, propanil, and its product formulation Stam Novel Flo®, was conducted including standard ecotoxicological endpoints as (1) growth inhibition of the freshwater green alga *Pseudokirchneriella subcapitata*; (2) immobilisation of the freshwater cladoceran *Daphnia magna*; and (3) avoidance behaviour of the earthworm *Eisenia andrei*. The EC50 values estimated for *D. magna* to propanil and Stam were generally within the toxicity range previously reported in the literature (propanil EC50 for *D. magna* within 1.2–5.0 mg/L). Nevertheless, these herbicides appear not to have affected the *E. andrei* terrestrial earthworms. Furthermore, even if differences in EC50 were noticed as compared to the control group, no difference between the toxic effect of the active ingredient and the product formulation was observed (Pereira et al., 2009). Mamdouh and colleagues, measuring

chromosomal abnormalities and the mitotic index determination in rat bone-marrow cells, tested the genotoxicity of tribenuron-methyl and the commercial product Granstar. They found an increased toxicity of the commercial product, compared with the active ingredient, probably due to the presence of additional hazardous components (Mamdouh et al, 2012). Danion and colleagues investigated the toxicity of pendimethalin and its commercial formulation Prowl 400, exposing Rainbow trout for 28 days to the active ingredient and to the commercial product. Thanks to the evaluation of haematological and immune parameters, as well the concentration of herbicides in flesh, they found a higher immunotoxicity for Prowl 400 than pendimethalin alone (Danion et al., 2012).

The toxicity of a common herbicide formulation, Treflan 4D® (active ingredient: trifluralin), was evaluated by Weir and colleagues exposing green frog (*Lithobates clamitans*) tadpoles in order to compare herbicide product formulation toxicity to technical-grade trifluralin. Treflan 4D®, showed an LC50 of 2.81 mg/l, and was found to be more toxic to tadpoles than trifluralin (LC50: 9.76 mg/l) (Weir et al., 2012). Similar results were obtained by Rocha and colleagues when testing Nortox® and the corresponding active ingredient diuron on *A. inaequalis*, *D. furcatus* and *S. trispinosa*. The LC50s were 20.27, 12.18, 27.90 mg/L for diuron and 15.52, 4.61, 10.48 mg active ingredient/l for Nortox®, for *A. inaequalis*, *D. furcatus* and *S. trispinosa*, respectively. Thus, based on these LC50 values, a higher toxicity for the product formulation vs active ingredient was observed (Rocha et al., 2018).

3.2.2. Insecticides

Avermectin, hexaflumoron, chlorfluazuron, chlorpyrifos, tebufenozide and their combinations with surfactants (Tween 80® and PEG6000®) were investigated by Li and colleagues with the aim to assess their comparative cytotoxicity (Li et al., 2015). The study showed that the relatively high cytotoxicity towards HePG2 and Tn5B1-4 cells caused by avermectin exposure was mitigated by Tween 80® and PEG6000®. The cytotoxicity of chlorfluazuron in Tn5B1-4 cells was also reduced by PEG6000 but enhanced by Tween 80. Furthermore, co-incubation with Tween 80 increased the cytotoxicity of chlorpyrifos and tebufenozide on HepG2 cells.

Differential cytotoxicity of various insecticides (pirimicarb, imidacloprid and acetamiprid) and their product formulations (Pirimor G®, Confidor® and Polysect Ultra®) was assessed on human secondary cell cultures by Mesnage and colleagues who concluded that all of the tested product formulations were far more toxic than their active ingredients (Mesnage et al.,

2014). In contrast, the ability of pirimicarb to induce micronuclei in CHO cells was found to be equivalent with that of its product formulation Aficida as reported by Soloneski and colleagues (Soloneski et al., 2015).

Amitraz, and its product formulation Azadieno® were investigated by Padula and colleagues who compared the genotoxic potential of the active ingredient to that of the product formulation in CHO cells. Azadieno® induced statistically significant genotoxic effect at lower concentrations than amitraz alone (Padula et al., 2012).

Azadirachtin-based pesticides (Neemix® and Bioneem®), formulated with neem tree extracts, and pure azadirachtin were studied by Goktepe and colleagues to compare their cytotoxic effects on a murine hybridoma cell line and on isolated oyster heart cells. The study indicated that the product formulations, but not azadirachtin, were toxic in both cell types in a dose-dependent manner (Goktepe and Plhak, 2003).

Cosenza and colleagues investigated the toxicity of chlorpyrifos, as a product formulation (Rid-a-bug) and as an active ingredient in three organic solvents, on nervous system development using midbrain micromass cells isolated from rats, and found that the product formulation was less toxic than the solutions of the active ingredient; however, the observed differences were not significant (Cosenza and Bidanset, 1995).

Rocha and colleagues observed a higher toxicity of the product formulation Furadan 350 SC®, compared to the active ingredient carbofuran (LC50: 1.382, 0.314, and 0.024 mg/l for carbofuran and 1.248, 0.253 and 0.041 mg/l for Furadan 350 SC® for *A. inaequalis*, *D. furcatus* and *S. trispinosa* respectively), except for *S. trispinosa* where the active ingredient carbofuran was more toxic than its product formulation (Rocha et al., 2018). Kitulagodage and colleagues observed similar findings when testing the product formulation Adonis 3UL® vs the active ingredient fipronil. The values of the estimated LD50 were 45.41 mg/kg and 310.2 mg/kg for Adonis 3UL® and fipronil, respectively. The toxicity of the pure insecticide resmethrin, and its common product formulation, Scourge®, have been compared by Key and colleagues on adult and larval estuarine grass shrimp (*Palaemonetes pugio*), in two types of tests: a 96-h static renewal aqueous test without sediment, and a 24-h static nonrenewal aqueous test with sediment. For resmethrin, the 96-h aqueous LC50 value for adult shrimp was 0.53 μg/l, and for larval shrimp was 0.35 μg/l. In the presence of sediment, technical resmethrin produced a 24-h LC50 value for adult shrimp of 5.44 μg/l, and for larval shrimp of 2.15 μg/l. When the Scourge® product formulation (18% resmethrin + 54% PBO) was adjusted for resmethrin, the product formulation was found to be more toxic to adult and larvae grass shrimp than the active ingredient, except for the larval sediment exposure. The

adjusted LC50 values for Scourgae® were 0.37µg/l for adult shrimp, and 0.07 µg/l for larval shrimp, for 96-h. The 24-h sediment test yielded an adjusted LC50 value of 16.12 µg/l for adult shrimp, and 14.16 µg/l for larvae (Key et al., 2005).

Nebeker and colleagues have studied the impact of gluthion and its product formulation Gluthion 2S® on survival and growth of *Pseudacris regilla* frog (Nebeker et al., 1998). It was found that the no observed adverse effect level (NOEL) and the lowest observed adverse effect level (LOEL) for Gluthion 2S® (0.07 ±0.06 mg /l and 0.17 ±0.09 mg/l, respectively) were much lower than for the active ingredient gluthion (0.98 ±0.06 mg/l and 3.60 ±0.35 mg/l, respectively). Furthermore, only Gluthion 2S® was tested on larvae of *Ambystoma gracile* and *Ambystoma maculatum* salamanders and comparable results as for the tree frog were reported. Nevertheless, these results could not be taken into account in the present review since the active ingredient gluthion was not tested on the same larvae (Nebeker et al., 1998).

Short-term exposure to a monomethyl carbamate insecticide, methomyl, and its product formulation Lannate, was studied by Pereira and colleagues, including standard ecotoxicological endpoints as (1) growth inhibition of the freshwater green alga *Pseudokirchneriella subcapitata* (OECD 2006); (2) immobilisation of the freshwater cladoceran *Daphnia magna* (OECD 2004); and (3) avoidance behaviour of the earthworm *Eisenia andrei* (ISO 2005). The product formulation Lannate and the active ingredient methomyl have shown the same toxicity to *D. magna* (formulation: 0.022–0.026 mg/l; active ingredient: 0.019–0.022 mg/l). The toxicity of the formulation was found to be lower (184 mg/l) for *P. subcapitata* compared with the active ingredient (108 mg/l). When exposed to Lannate; however, *E. andrei* significantly avoided the soil contaminated with the product formulation at lower concentrations than with the active ingredient methomyl (Pereira et al., 2009).

The genotoxicity of pyrethroids, such as pypermethrin, cyphenothrin, deltamethrin and permethrin were investigated by Demir and colleagues, alone or in combination with piperonyl butoxide (PBO). The authors conclude that PBO looks not having any synergic effect in combination with the active ingredients, when tested on Drosophila Melanogaster (Demir et al, 2014). Moretti and colleagues compared the mutagenic effects of deltamethrin and its product formulation Decis, and found some differences in the data obtained, confirming that product formulations may contain additional hazardous compounds (Moretti et al., 1997). The same conclusion was obtained by Abbassy and Mossa, comparing the

toxicity of active compounds deltamethrine and cypermethrin with their respective formulation Sparkle (25% active ingredient) and K-Othrin (2.5% active ingredient) in rats (Abbassy and Mossa, 2012). Similar results have been found by Lechner and Abdel-Rahman, who studied the teratogenic effect of malathion, carbaryl and their commercial formulation Ortho. Furthermore, when the carbaryl and malathion were used together in a mixture, increases in hazards producing maternal toxicity were observed (Lechner and Abdel-Rahman, 1984).

3.2.3. Fungicides

Cytotoxic effects of three fungicide ingredients (tebuconazole, epoxiconazole and prochloraz) in relation with their product formulations (Maronee®, Opus® and Eyetak®) were comparatively assessed by Mesnage and colleagues using secondary human cell lines. Exposure to all the product formulations resulted in lower viability parameters of HEK293, HepG2 and JEG3 cells if compared to exposure to the individual active ingredients (Mesnage et al., 2014). Cytogenetic effects of zineb and its product formulation Azzurro® were examined by Soloneski and colleagues in three studies with different endpoints and conclusions. One of them reported that Azzurro® is able to induce DNA damage in CHO cells at significantly lower concentrations than the active ingredient zineb (Soloneski et al., 2002). In the second study, neither cytotoxicity, nor increase of micronucleus frequency was observed when human mononuclear leukocytes were exposed to the pure or formulated compound (Soloneski et al., 2001). In the third study, zineb and Azzurro® increased the frequency of micronuclei when CHO cells were exposed within the 1–5 μg/ml and 1–10 μg/ml concentration ranges to zineb and Azzurro®, respectively, and zineb more actively induced cytotoxic effects in CHO cells than Azzurro® by decreasing both cell viability and succinic dehydrogenase activity (Soloneski et al., 2015).

4. Discussion

In this systematic review, 36 studies have been identified dealing with the evaluation of the toxicity of product formulations compared to active ingredients. Among which, 24 studies found that at least one pesticide product formulation possesses higher toxicity than its corresponding active ingredient. Only 8 studies reported reduced toxicity of PPFs, which might be due to a potential antagonistic effect of the co-formulates and the active ingredient. Even if not all the active ingredients and the formulations reviewed in this article are approved in the European Union, all these findings taken together underpin the legitimate demand of the European Commission for a more stringent regulatory regime governing the authorisation of PPFs by requiring the producing companies to provide "*information on safeners, synergists and co-formulants*". Recognizing the inadequacy of past testing requirements for assessing the health hazards of pesticide products based mainly on the evaluation of the active ingredients, the toxicity of PPFs is currently to be subjected to a more complex evaluation and authorisation process (European Commission, 2013). It has been a general perception for a long time that the compounds present in product formulations (other than the active ingredients) are biologically 'inert'. The findings of the current review contradict this notion as several articles have identified the potential toxic effects of these 'inert' compounds, which are, among others, surfactants (e.g. POEA and APG) (Defarge et al., 2016) or even neurotoxic solvents, like xylene and mesitylene (Freydier and Lundgren, 2016). An illustrious example of the changing perception in the recent years is the banning of the co-formulant POEA from glyphosate formulations in 2016 (European Comission, 2016). In this context, testing of the complete final product formulation should be considered in addition to the testing of the active substances and co-formulants separately, and that should be the case also if the formulation is subject to changes.

As to the hazards arising from the usage of these formulations, besides the obvious work-related exposures, depending on the nature and behaviour of co-formulants, bio- and photodegradation, handling crops and food contaminated with these chemical mixtures also carry health risks. Adsorption, biodegradation, photodegradation of pesticides by sunlight as well as the levels of pesticide residues in various crops are mainly considered for the active ingredients; however, reactive intermediates can also be formed from co-formulants, and the effects of these intermediates are not always taken into account in the risk assessment process. As the fates of active ingredients in the environment and in the human body are more completely described than those of formulations, the possible exposure arising from the

degradation products of co-formulants mostly remains hidden. Surfactants, adjuvants, non-evaporating viscous stickers and other commonly used additives in pesticide formulations require further investigation individually in order to assess their potential hazards during and after environmental transformation. The possible newly emerging chemical interactions due to natural processes can alter and enhance the known toxicological endpoints causing unexpected environmental and human health effects. Currently, components other than the active ingredient in a formulation are not taken into consideration when setting the maximum residue level (MRL) for a certain pesticide (European Comission, 2018). Moreover, measuring the levels of the degradation products is not legally binding, which makes it more difficult to keep control over pesticide exposure scenarios for the general population.

The identified clear differences in the harmfulness of active ingredients and product formulations contribute to growing awareness of the complexities of the toxicity of chemical mixtures in general, even if that was not the primer intention of the different programs currently under development. For instance, in the recent past, the Agency for Toxic Substances and Disease Registry (ATSDR) has launched a "Chemical Mixtures Program" to determine the health impact of exposure to combinations of chemicals (generally, active ingredients/compounds) by (1) identifying the mixtures of highest concern to public health, (2) estimating the joint toxic action of these chemicals through assessment and laboratory methods, and (3) developing of new methodologies for evaluating the health effects of mixtures (ATSDR, 2010).

Dealing with mixtures also poses substantial regulatory challenges, with numerous pertinent EU and national regulations. In the European Directive 396/2005 the European Food and Safety Authority (EFSA) was appointed to be responsible for establishing the methodology for risk assessment of chemical mixtures. It is stated among other things "…*It is also important to carry out further work to develop a methodology to take into account cumulative and synergistic effects. In view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health, MRLs should be set after consultation of the European Food Safety Authority*…." . Since 2005, EFSA has published 4 Opinions and 1 Guidance on how to perform risk assessment for pesticide mixtures. The full methodology was discussed during an EFSA information session organized to discuss the methodology with the stakeholders (EFSA, 2014). The Joint Research Centre of the European Commission (JRC) has also published several reports on assessment of mixtures, that advocate a new test strategy to define the relevant mixtures

(Bopp et al., 2015). EFSA takes pesticides as a point of departure to further develop strategies of evaluating mixtures.

Several different guidance documents on risk assessments for mixtures have been published recently, each focusing on a specific group of compounds or type of assessment. Examples include the guidance on aquatic risk assessment under REACH (Bunke et al., 2014), the assessment of mixture effects of biocides (ECHA, 2014) and guidance produced by EFSA on mixtures of pesticides (EFSA, 2013).

In the context of the REACH Regulation (No 1907/2006), guidance has been developed concerning the assessment of multiple sources of exposure to a single substance and in specific cases to the assessment of several closely related and similarly acting substances, such as different salts of the same metal or a number of closely related derivatives of organic substances (see for example ECHA, 2016, section E.3.5). Whilst this gives some scope to assess possible adverse effects associated with known combinations, it does not address possible concerns associated with exposure to unknown mixtures.

Regarding mixtures of pesticides, Regulation 396/2005/EC on maximum residue levels of pesticides in or on food and feed of plant and animal origin highlights the importance of further work to develop a methodology to take into account cumulative and synergistic effects. The regulation notes that "*In view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health, maximum residue levels should be set after consultation of the European Food Safety Authority*".

In response to this mandate, the European Food Safety Authority (EFSA) has undertaken a programme of work on mixtures risk assessment (see EFSA's webpages on mixtures). EFSA's activities in the area of mixtures have included: (1) the development of approaches for assessing combined exposure to multiple pesticides in bees (Spurgeon et al., 2016); (2) a scientific opinion on the identification of pesticides for inclusion in cumulative risk assessment groups on the basis of their toxicological profile (EFSA, 2013); and (3) guidance on probabilistic modelling of dietary exposure, applied to pesticide residue mixtures from the triazole group (Boon et al., 2015). In addition, under an initiative called MixTox, EFSA has established a working group (EFSA, 2016) of experts to develop guidance aimed at harmonising methodologies for assessing human, animal and ecological risks resulting from exposure to multiple chemicals. Recently, this guidance presenting a tiered approach of how this issue could be addressed was published by the European Food and Safety Authority (EFSA, 2019).

The ongoing activities demonstrate the need for the systematic toxicological assessment of complex chemical products and the findings of this systematic review clearly support the initiative and advise for further inclusion of formulations, such as PPFs, in mixtures evaluation.

4.1. Limitations

Given the heterogeneity and the relatively limited number of studies evaluated, we are unable to unequivocally ascertain the list of product formulations with pronounced adverse effects. Comparative analysis of technically valid studies investigating commercial pesticide formulations or active ingredients separately has not been performed in this review, which could further contribute to the knowledge on the differences of toxicity between PPFs and active principles. In addition, the equivalent concentrations of the active substances as dosed alone or within a formulation were considered to conclude on the differential toxicity observed. For future research, we advise that equi-effective concentrations should be taken into account when designing the studies in order to have a more accurate conclusion on the relative toxicity of active ingredients and commercial formulations.

Potential conflict of interests were identified in three studies as follows: (1) Flaherty and colleagues carried out the study in the laboratory of a pesticide-producing company (Monsanto) that manufactured the tested herbicide and it would therefore be in the interest of the authors to represent the product as safe (Flaherty et al., 1992); (2) Jonathan G. Lundgren had commercial links with Monsanto, by selling predatory beetles for safety research, and from which he received honoraria for speaking at Beyond Pesticides and various Conservation Agriculture and Pollinator-related groups but their results seem to be scientifically reliable (Freydier and Lundgren, 2016); and (3) in the study of Hayes and colleagues the tested compounds (i.e. S-metolachlor and Bicep II Magnum) have been donated by Syngenta Crop Protection U.S and the Novartis, Syngenta Crop Protection, Ecorisk Inc., and members of the Atrazine Endocrine Ecological Risk Assessment Panel of Ecorisk Inc have commented, criticized and encouraged the authors, which might shade some doubts as for their findings concerning a reduced toxicity of the product formulation as compare to the active ingredient (Hayes et al., 2006).

5. Conclusion

The results of this review suggest that the toxicological hazards posed by complex chemical mixtures such as formulated pesticide products should not be underestimated. For instance, we could highlight from the reviewed articles that glyphosate formulations can exert more pronounced cytotoxic and endocrine disruptive effects compared to that of pure glyphosate. Thus, ignoring the possible risks deriving from the interaction between the active and other ingredients of a commercial pesticide product formulation can result in the misinterpretation of its toxicological profile. Therefore, we recommend that all the different product formulations should be fully assessed during the pesticide pre-market evaluation process, and we also advocate the initiation of an effective post-market vigilance system to systematically monitor the real-life impacts of the use of PPFs on human and animal health and on the environment as a whole.

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- Several pesticide product formulations have different toxicities than their active ingredients.
- Interactions of ingredients in pesticide product formulations should not be ignored.
- Formulations should be fully assessed in the pesticide pre-market evaluation process.
- Post-market vigilance systems should be used to monitor real-life impacts of pesticide product formulations.

Declaration of interests

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☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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