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Aquatic ecotoxicity of glyphosate, its formulations, and co-formulants: evidence from 2010 to 2023

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Abstract

Glyphosate (GLY), the most widely used herbicide active ingredient (AI) in the world, is frequently detected in aquatic environments where it can affect non-target organisms. Globally, more than 2000 commercial GLY-based herbicides (GBHs) are used to control weeds. Non-target organisms are exposed to complex pesticide formulations under real environmental conditions, but the co-formulants contained in GBHs are classified as so-called inert and inactive ingredients in terms of their biological effects. The main objective of this comprehensive review is to compile the results of aquatic ecotoxicological studies on the side-effects of GLY, GBHs, and their formulating agents. Based on the results demonstrated for a variety of plant and animal aquatic organisms, oxidative stress appears to be a major trigger for these adverse effects, affecting the integrity of DNA and other biochemical functions. Furthermore, there is evidence of impairment of various physiological and behavioral functions. Adverse effects of GLY and GBHs have been observed even at very low concentrations. There are also differences in the sensitivity of the aquatic organisms tested, even with similar lifestyles, habitats or identical taxa. The studies typically investigate the short-term effects of a single exposure to GLY/GBH on a single species, whilst in reality multiple applications of GBHs together with other pesticides are common during a cropping cycle. Moreover, the interactions between GLY/GBHs and other aquatic contaminants are rarely studied. Higher toxicity of GBHs compared to GLY alone has often been observed, demonstrating that co-formulants can be highly toxic on their own and markedly increase the toxicity of the GBH formulation. The possible impurities in GBHs, such as heavy metals, can cause additional problems for the environment and food safety. The widespread and massive use of GBHs leads to increased exposure and environmental hazards. In addition, the need for a revision of the risk assessment system is emphasized. According to the results of aquatic ecotoxicological studies, the current use and pollution of the aquatic environment by GLY/GBHs is highly problematic and cannot be considered environmentally sustainable. It is, therefore, necessary to at least tighten the permitted forms of use.

Keywords Glyphosate, AMPA, POEA, Roundup, Co-formulants, Aquatic ecotoxicity, Algae, Daphnia, Fish, Amphibians

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Introduction

Over the last decade, an increasing number of scientific studies have investigated the effects of the most widely used herbicide active ingredient (AI) glyphosate (GLY) on non-target organisms [1–3]. GLY (*N*-(phosphonomethyl)-glycine) is a phosphonomethyl derivative of the natural amino acid glycine [4]. Cultivation of GLY-tolerant (GT) genetically modified (GM) crops such as soybeans and maize in North and South America has led to a massive increase in the use of GLY-based herbicides (GBHs) and they have become the most widely used herbicide formulations in the last decade [5–8], despite their known water-polluting properties and the emergence of GLY-resistant weeds [1]. Based on a European survey, GBH sales were estimated at 44,250 tonnes of AI, while the average GLY use in 2017 was about 0.24 kg AI ha^{-1} [9]. The global market of GBHs was estimated at 4438.5 million USD in 2020 [10], but it is very difficult to find accurate and up-to-date data on global use and sales of GBHs because detailed sales data are withheld as commercially sensitive information [11].

GLY exerts its herbicidal activity by inhibiting 5-enoylpyruvylshikimate-3-phosphate synthase (EPSPS) of the shikimate metabolic pathway. This leads to a blockage of the biosynthesis of essential aromatic amino acids and consequently to plant death. The shikimate metabolic pathway is present in all plants and thus GLY acts as a non-selective broad-spectrum herbicide. However, the shikimate pathway is also present in most fungi and some bacteria, but it is absent in animals [12]. Therefore, the application of GBHs as non-selective herbicides not only causes the death of plant species, but can also negatively impact fungal and bacterial populations [13, 14]. In GBHs, different salts of GLY such as GLY-isopropylammonium salt (GLY-IPA), GLY-trimethylsulfonium salt or GLY-diammonium salts are used to enhance the solubility of the AI [15, 16]. In addition to GLY salts, various co-formulants are included in commercial GBH formulations. The key property of co-formulants is to act as surfactants enabling effective wetting and penetration of the plant cell wall, thereby permitting the AI to exert its herbicidal action [17]. For example, the use of POEA (a mixture of polyethoxylated tallow amines sold under product names such as MON 0818) in GBHs promotes GLY penetration into the plant cell [18]. Crucially from an environmental impact perspective, in addition to their designed herbicidal activity, GBHs have also been found to exert direct insecticidal effects on numerous non-target arthropod species including lacewings (*Chrysoperla carnea*) [19], spiders (e.g., *Pardosa* spp.) [20–22], mosquitos (*Aedes aegypti* larvae) [23], and pollinators such as bees (e.g., *Megachile* spp. and *Apis mellifera*) [24–26]. Whether these insecticidal effects of GBHs are due to GLY, the co-formulants, or a combination of the two cannot yet be accurately determined because most studies have not conducted a comparison between GLY and GBHs.

Co-formulants in commercial pesticides are considered to be inactive components in terms of the primary biological action of the formulation. As a result, co-formulants are usually listed as "inert" and their identity withheld on the packaging. Therefore, a simpler environmental risk assessment (ERA) has been deemed sufficient for co-formulants compared to AIs for regulatory purposes [27, 28]. Furthermore, regulatory authorities acquire data about co-formulants through individual stand-alone studies rather than considering them within formulations. Consequently, the differential effects of commercial pesticide formulations on ecosystems and humans are typically not due to the inherent attributes of co-formulants as independent components, but to how these co-formulants modify the toxicity of AIs [29]. However, numerous studies spanning many years have demonstrated the high toxicity of co-formulants and also the increased combined toxicity of AIs and co-formulants in various commercial pesticide formulations of all types (herbicides, insecticides, fungicides) compared to the toxicity of individual AIs. This applies to POEA, which is used as a co-formulant in GBHs [30–32], as well as its alternatives [33, 34]. Due to incriminating scientific evidence, the use of POEA in GBHs has been banned in the European Union (EU) by Regulation 2016/1313 [35].

Regulation of commercial pesticide formulations in the EU is based on a detailed and harmonized two-tier system [36]. AIs are registered at the EU community and managed by the European Commission, whilst commercial pesticide formulations are approved at the Member State level [37]. Several studies indicate that pesticide authorization needs to be revised [19, 38], including the re-evaluation of current testing systems during the registration process [19]. The approval and ERA for commercial pesticide formulations consider certain hazards but do not act through central regulation and restrictions. Moreover, EU Member States governments or their affiliated governmental organizations are required to take into account the positions of all stakeholders, including industry and also patent holders, during the risk assessment procedure [39].

Originally, non-selective GBHs were used exclusively for pre-emergence weed control. However, with the launch of GLY-tolerant genetically modified (GT GM) crops in 1996 (which are not authorized for cultivation in the EU) and the practice of pre-harvest desiccation in agriculture, the use of post-emergence GBHs has risen exponentially, leading to a vast increase in use over the last 25 years [6, 40, 41]. As a consequence of its escalating

and excessive global use, GLY has become a ubiquitous pollutant in aquatic ecosystems [42]. Generally, GLY is directly sprayed onto crop fields not only for weed control but also for no-tillage farming, where a significant proportion is taken up by plants or enters the soil. In soil, GLY may be transported by surface water runoff, adsorbed to soil particles, enter groundwater by infiltration, or enter surface waters. The occurrence and concentration of GLY in the aquatic environment after its application are highly dependent on abiotic (e.g., pH, suspended materials, hydrological conditions), biotic (e.g., microbial composition), and climatic conditions (e.g., rainfall frequency and intensity) [43–45], in addition to the timing and frequency of pesticide treatments [44, 46]. In addition, GBH co-formulants such as POEA, similar to GLY, have been found to be widely distributed in the Midwest of the USA (e.g., Iowa, Illinois, Missouri) [47], where agricultural areas are large and the cultivation of genetically modified GT crops is concentrated [48]. Furthermore, POEA has been shown to persist in soil along with GLY and its primary metabolite aminomethylphosphonic acid (AMPA) [47, 49, 50], and can enter natural waterways [49, 51, 52]. Thus, GLY and co-formulants coexist in soil and water courses, although their combined toxic effects on the environment poorly are poorly understood.

Various aquatic organisms are directly or indirectly exposed to the harmful effects of GBH residues. To determine the potentially harmful effects of chemical contaminants on non-target aquatic organisms, a specific group of organisms is usually used in ecotoxicological studies to ensure environmental relevance. Examples of these test organisms include aquatic unicellular plant organisms (e.g., algae), aquatic invertebrates (e.g., water fleas) and vertebrates (e.g., fish). As part of the EU authorization process for pesticide formulation, an AI, safener or synergist shall only be approved, if the results of the risk assessment confirm acceptable or no risks [36]. As part of the tiered risk assessment for pesticides, the ecotoxicological test methods for assessing aquatic ecotoxicity are covered and summarized in the corresponding technical guidance document of the European Food Safety Authority (EFSA) [53]. The authorities of the EU Member States are responsible for ensuring the safety of pesticide formulations on the basis on the requirements of Regulation (EC) 1107/2009 [36].

Currently, the occurrence of GLY in surface waters is a global phenomenon, especially in regions where preharvest desiccation practices are widespread and the cultivation of GT GM crops takes place, so that the exclusive use of GBHs is extremely high. As a result, GLY contamination levels in surface water can reach up to 5200 μg l^{-1} [39, 54, 55]. The increased use GLY through desiccation or post-emergence application to GT GM crops generally increases the release of GLY and its co-formulants into the environment, which in turn leads to increased exposure. Such exposure can occur in any aquatic system, so increased toxicity can be exerted on all aquatic organism concerned, from aquatic microorganisms, algae and plants to aquatic invertebrates and vertebrates. Due to its amphoteric properties, GLY has both acidic and basic properties and is therefore highly soluble in water, although its detection in various environmental samples and matrices is difficult [56, 57]. In the past, GLY was not part of general pesticide monitoring programs, so environmental concentrations of GLY and its metabolites were underestimated. However, with advances in detection methods, GLY has been shown to be a ubiquitous environmental pollutant [58]. The primary metabolite of GLY, AMPA, is more mobile than the parent compound [59] and is also frequently detected in various environmental matrices such as groundwater, surface waters, soil, and air [39, 60–63]. However, it should be kept in mind that the presence of AMPA in environmental matrices such as groundwater, influents, or sewage sludge is not exclusive due to GLY metabolism, as it can also originate from phosphonate detergents used in various detergents [64].

Surveys of GLY residue levels in various water samples have shown a wide range of variation [39]. According to the U.S. Geological Survey, GLY and/or AMPA were detected in 59% of the 470 surface water sites analyzed, while the occurrence of the measured compounds in groundwater samples was less frequent (8.4% of 820 sample sites). AMPA was generally detected more often than GLY in the samples analyzed [51]. In surface waters collected in the Rio de Janeiro region, the GLY level detected was 2.6–10.1 μg l^{-1} (in >40% of the samples analyzed) [65]. In Argentina, the average concentration of GLY and AMPA detected in surface water samples was in the range of 17.5–35.2 μg l^{-1} and 0.6–2.1 μg l^{-1}, respectively [66]. However, maximum GLY and AMPA concentrations of up to 258 μg l^{-1} and 5865 μg l^{-1}, respectively, were detected in the groundwater and surface water samples [52].

Based on the European monitoring studies over the past decade, the extent of GLY contamination in surface waters in the EU appears to be lower (typical GLY concentrations detected were between 0.05 and 0.85 μg l^{-1}), although residues are consistently present [39]. In a monitoring study of sub-catchments with different land use (agricultural, urban) in Switzerland, the maximum GLY concentration of 4.15 μg l^{-1} was detected in the sampled water at peak discharge during storm events throughout the year, so that the seasonal concentration and occurrence of GLY cannot be explained by agricultural use

alone [67]. According to a Dutch database with information on 161 sampling points, 90% of the surface water samples analyzed in 2020 contained GLY, while in 2019 only one sample contained GLY above 77 µg l^{-1} (152 sampling points) [68]. In Hungarian, Swiss, and Italian water samples, the GLY concentrations detected were between 0.035 ng ml^{-1} and 96 µg l^{-1} [39, 55, 69, 70]. However, GLY and AMPA concentrations in wastewater after rainfall can reach up to 384.9 µg l^{-1} and 47 µg l^{-1} [71]. The observed differences can primarily be explained by different agricultural locations, characteristics of the catchment area and natural precipitation conditions, which lead to different runoff and leaching of AI into surface waters [55]. Furthermore, co-formulants are also found in environmental compartments, although they are generally not monitored [48, 49], which may have adverse effects on non-target organisms [72, 73]. In summary, numerous scientific publications have demonstrated the highly unpredictable risks of GLY to aquatic ecosystems [39, 74, 75].

The objective of this review is to present and summarize pertinent information reported since the EU Commission Directive 2010/77/EU on the ecotoxicological adverse effects of GLY, GBHs, and their formulating agents on various non-target organisms and communities. This study not only presents the aquatic ecotoxicological concerns related to GLY/GBHs, but also summarizes the combined effects of GLY and GBHs with other aquatic pollutants (e.g., other pesticide residues, heavy metals, nano- and plastic particles) or pathogens. Systematic searches were conducted in scientific databases including Science Direct, Scopus, Web of Science, and other relevant databases. In addition, the references cited in the selected studies were also taken into account when necessary. Furthermore, non-public ecotoxicological studies financed and commissioned by the industry, that were not included in the application dossiers for re-approval [76] were excluded from evaluation. In total, an extensive reference database of more than 500 scientific publications dealing with the ecotoxicological aspects of GLY or GBHs was assessed. This review focuses specifically on the articles relating to aquatic ecosystems.

Ecotoxicity to aquatic organisms and ecosystems

Aquatic organisms are highly exposed to pollution as contact with waterborne xenobiotics is unavoidable. The ecotoxicity of GLY and GBHs has been studied in numerous aquatic organisms, including various algae species [77, 78], small planktonic crustacean such as *Daphnia magna* [79], molluscs [80], fish [81], and amphibians [82] (Fig. 1). Due to the long-lasting toxic effects of GLY, it is classified by the European Chemicals Agency ECHA as toxic to aquatic life (aquatic chronic 2; H411) [83]. However, a number of studies indicate that even at low concentrations GLY exhibits a toxicity to the aquatic environment that would justify a category 1 classification for chronic and even acute aquatic toxicity [81, 84]. In turn, GBHs are very rarely approved for use in the

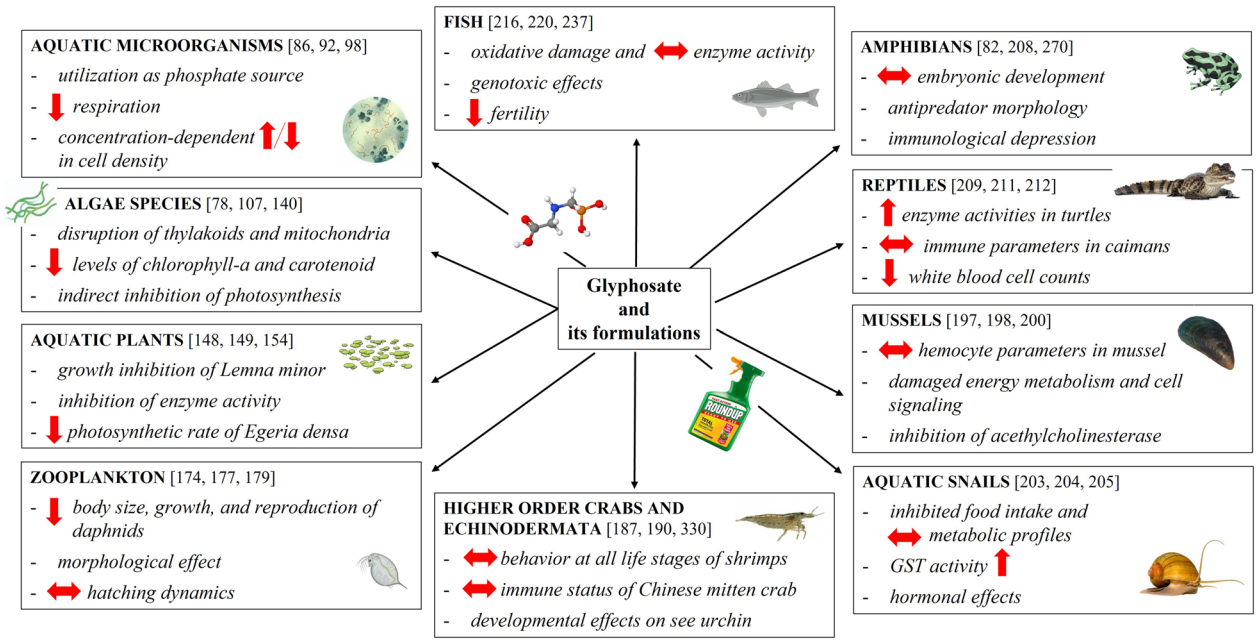

Fig. 1 Main ecotoxicological effects of glyphosate and its commercial formulations. Figure created with BioRender. Upward red arrows: increase; downward arrows: decrease; horizontal bi-directional arrows: alteration

aquatic environment, yet GLY, its metabolite AMPA and co-formulants of GBHs are frequently detected in surface waters worldwide [85]. Moreover, as mentioned above, increased pollution levels by GLY residues due to the increased application of GLY during desiccation or on GT GM crops can affect all aquatic organism in the affected water bodies. This is a clear example of an increased likelihood of an existing hazard occurring due to the increased exposures to the aquatic pollutant.

Effects on aquatic microorganisms

Based on the scientific literature, the changes in aquatic microbial communities can be determined using direct (e.g., cell number, density, composition) and indirect (e.g., extracellular secretion, rate of leaf-litter breakdown, respiration) endpoints following GLY exposure [86–90]. As little as 10–100 µg l^{-1} GLY can cause direct adverse effects on most bacterioplankton taxa [91] and changes in the structure of freshwater microbial communities [87]. However, the effects on aquatic bacterial communities were usually observed at higher concentrations (≥ 2.5 mg l^{-1}), resulting in a loss of biodiversity [88] (Table 1). In addition, a reduced decomposition rate of leaf-litter was observed in natural streams, possibly due to the negative effects of GLY (710 µg l^{-1}) on the microbial community [90]. In artificial microcosms, GLY had no significant impact on the composition of the microbial community in water [92, 93], but community patterns of transcription were significantly altered [92]. The observed effects could be mainly due to the utilization of GLY by microorganisms as a phosphate source [92]. Furthermore, selective growth of different bacterial groups has also been demonstrated [94].

In aquatic environments, biofilms colonizing various artificial and natural substrates are compact communities of photoautotrophic (algae species) and heterotrophic microorganisms (bacteria, fungi, protozoa) embedded in their extracellular polymeric substance (EPS) secretions [89]. This EPS matrix is mainly composed of polysaccharides, proteins, lipids, nucleic acids and lectins, which can serve as sorption sites [95]. Scanning electron microscopy has revealed the intensive EPS production, primarily through secretion by heterotrophic microorganisms in freshwater biofilm communities after exposure to 100 µg l^{-1} GLY, particularly in the presence of the GBH co-formulant POEA [87]. This indicates a protective mechanism of bacterial and algal species in natural biofilms to remove and reduce the harmful effects of contaminants. Furthermore, GLY can affect the metabolic processes of bacteria and algae in biofilm communities [96]. The effects of GBH even at very low concentrations of 10 µg l^{-1} on the composition of the microbial community were significantly dependent on temperature. However, the effects of multiple stress factors on the microbial composition in water and sediment were completely opposite [97]. GLY at a high concentration of 2.54 g l^{-1} caused a significant reduction (−47%) in the respiration of heterotrophic species in biofilm communities [86]. One type of Roundup GBH reduced the cell density of planktonic *Pseudomonas aeruginosa* under aerobic conditions, whereas planktonic anaerobic growth was increased in the presence of GLY (from 84.5 mg l^{-1}) [98]. Furthermore, a concentration-dependent low growth of *P. aeruginosa* biofilms was also observed [98].

Based on a study conducted on the luminescent marine bacteria *Vibrio fischeri* and other test organisms such as crustaceans and plants, it was found that quaternary ammonium salts (e.g., diisopropylammonium chloroacetate) could be a safer alternative to GLY as they have lower toxicity but show comparable or slightly greater herbicidal activity compared to GLY [99]. However, the potential toxic effects of these quaternary ammonium salts on other non-target organisms remain to be investigated. Compared to *Daphnia magna*, *V. fischeri* was found to be nine times more sensitive to the toxic effects of Roundup formulations [100]. Moreover, aquatic test organisms were more sensitive to GBHs than soil microbial strains although a direct correlation between the toxicity of the formulations and the presence of POEA could not be demonstrated [100]. GLY and AMPA showed less negative effects in experiments with *Tetrahymena pyriformi* compared to *V. fischeri*, but with GLY displaying higher toxicity than AMPA in all cases [101]. However, no effects of Roundup on the aggregation behavior and cell morphology of *Tetrahymena thermophila* were observed, proteomic changes were indicated after GBH exposure (77.5–171 mg l^{-1}) [102]. Monitoring of free-living pelagic and benthic biofilm-associated bacterial communities in microcosms revealed a transient increase in total cell number and bacterial diversity of pelagic bacterial communities in the water column due to the presence of GLY, while biofilm communities were less affected [103]. Various co-formulants can also be used as nutrient sources by bacterial communities in freshwater biofilms, especially under nutrient-poor conditions. For example, non-ionic tallow-based alkylbis(2-hydroxyethyl) amines can be utilized as carbon and energy sources by various *Pseudomonas* species during their growth [104].

Effects on algae species

The identification of potential harmful effects on non-target plant organisms is an essential part of the ecotoxicological evaluation of herbicides. Based on the available ecotoxicological studies, the various adverse effects of GLY have been detected at much lower concentrations (1–100 µg l^{-1}) on phytoplankton communities compared

Table 1 Effects of glyphosate, its derivatives, co-formulants, and/or its formulated herbicide products on aquatic microorganisms reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
Bacterioplankton communities	^{13}C-labelled GLY[a]	10 and 100 µg l^{-1}	6 d[b]	Bacterial abundance, composition	No effects on abundance, altered bacterial diversity	[91]
Bacterioplankton communities	GLY	0–15 mg l^{-1}	43 d	Bacterial density, diversity, composition	Changed community structure (15 mg l^{-1}, no taxonomical effects	[88]
Benthic sediment microbial communities	GLY, AMPA[c]	0.07–7 mg l^{-1}	2 h[d] and 14 d	Diversity, composition	No effects on microbial communities	[93]
Microbial communities	GLY (pure 99.5%)	2.5 mg l^{-1}	15 d	Photosynthetic pigment content, transcriptomic analyses	Higher chl-a[e] content, no significant effect on the microbial composition, functional changes in microcosms	[92]
Bacterial community	GLY	6.09 and 0.9 mg l^{-1}	10 d	Bacterial diversity and composition	Bacterial community changes	[94]
Freshwater biofilm communities	GLY-IPA[f], Roundup Classic	100 µg AI[g] l^{-1}	35 d	Structural analysis	Intensive EPS[h] production	[87]
Microbial communities	GBH[i]	10 µg l^{-1}	5 months	Microbial community composition, assembly	Temperature-dependent and habitat-specific effects on microbial community assembly	[97]
Biofilm communities	GLY-IPA	0.25–2.54 g l^{-1}	22 d	Biofilm function and biomass	Decreased respiration (2.54 g l^{-1})	[86]
P. aeruginosa (planktonic, biofilm)	Roundup	0.845–1690 mg l^{-1}	24 h	growth	Reduced (aerobe) or increased (anaerobe, ≥ 84.5 mg l^{-1}) planktonic growth, concentration-dependent biofilm growth	[98]
V. fischeri, crustaceans	Quaternary ammonium salts	81.9% screening tests	15 min–6 d	EC_{50}[j]	No acute toxicity	[99]
V. fischeri, *D. magna*	GLY-IPA, GBHs (Roundup Max, Roundup Quick)	Stock solutions GLY-IPA and Roundup Max: 6.8 g a.e.[k] GLY l^{-1}, Roundup Quick: 7.2 g a.e. GLY l^{-1}	*V. fischeri*: 30 min, *D. magna*: 48 h	EC_{50}	Significant differences in EC_{50} values and the sensitivity	[100]
V. fischeri, *T. pyriformis*	GLY acid, AMPA	Screening tests	*V. fischeri*: 30 min, *T. pyriformis*: 9 min–2 h	*V. fischeri*: EC_{50}, *T. pyriformis*: esterase activity, IC_{50}[l]	*V. fischeri* more sensitive, GLY more toxic	[101]
T. thermophila	Roundup	100–300 mg l^{-1}	24–96 h	Growth, aggregation Behavior, cell morphology, proteomic analysis	96-h IC_{50} = 171 mg l^{-1}, no effects on aggregation behavior and cell morphology, proteomic alterations (77.5–171 mg l^{-1})	[102]
Pelagic and biofilm-associated bacterial communities	GLY	13.94 mg l^{-1}	140 d	Total cell count	Significant increase after GLY addition, biofilm communities less affected	[103]

[a] Glyphosate, [b]day, [c]aminomethylphosphonic acid, [d]hour, [e]chlorophyll-a, [f]GLY-isopropylammonium salt, [g]active ingredient, [h]extracellular polymeric substances,[i]GLY-based herbicides, [j]50% effective concentration, [k]acid equivalent, [l]half-maximal inhibitory concentration

to detectable GLY concentrations in surface waters. In many cases, however, the effects are only seen at much higher test concentrations (Table 2). A 48-h exposure to a Roundup GBH resulted in a significant reduction in growth and an increase in cell size of the unicellular green algae *Selenastrum capricornutum* with a 96-h EC_{50} value of 15.60 mg l^{-1} [78]. The most notable toxic effects were observed on the ultrastructure of exposed cells, including disruption of thylakoids and mitochondria, lipid accumulation, increased size and number of starch granules, and formation of electrodense bodies [78]. Larger cells of *Scenedesmus vacuolatus*, increased size of vacuoles and changed the stacking pattern of thylakoids after a 96-h exposure to the GBH Glifosato Atanor (containing 48% GLY as isopropylamine salt) at the range of 6–8 mg l^{-1} with an addition of 2.5% of the surfactant alkyl aryl polyglycol ether [105].

Moreover, altered oxidative stress parameters were also demonstrated. The observed effects can be attributed to an oxidative stress response resulting from the toxic mechanisms of the GBHs studied [105]. Furthermore, exposure to GBH Factor 540R affected the structure and functional properties of the freshwater phytoplankton community collected from agricultural areas in a concentration-dependent manner [106]. As a result, lower diversity (≥ 5 µg l^{-1}) and pigment content (chlorophyll-a (chl-a) and carotenoids, ≥ 1 µg l^{-1}) and altered biochemical and physiological parameters such as lipid peroxidation, antioxidant activity of catalase, superoxide dismutase (SOD), ascorbate peroxidase (≥ 500 µg l^{-1}) [106], in addition to photosynthetic parameters (≥ 10 µg l^{-1}) [107] were observed. It is worth noting that different algal and cyanobacterial species exhibit different sensitivity to GLY, even within the same taxa, resulting in significant differences in reported toxicity levels [108–110]. For instance, *Pseudokirchneriella subcapitata* showed a 72-h EC_{50} range of 24.7–41 mg l^{-1} [30, 111], while *Desmodesmus subspicatus* showed a 72-h EC_{50} range of 72.9–166 mg l^{-1} [112–114]. Exposure to a GBH (Roundup PowerFlex—4 mg a.e. GLY l^{-1}) reduced algal community diversity by 6%, and the decreasing effect was much more pronounced at the higher test temperature (20 °C vs. 15 °C) [115]. However, the density of algae was not affected by the treatments. In addition, an interaction between herbicide and temperature was observed, indicating a temperature-specific effect of GBH on the diversity of algal community [115]. The growth of *Chlorella vulgaris* was promoted after individual and combined exposure to GLY and AMPA (≤ 0.5 mg l^{-1}). In contrast, inhibition of algal growth was observed at the higher concentration tested (≥ 5 mg l^{-1}) [116]. However, the inhibitory effect of AMPA was only demonstrated in the presence of GLY [116].

GBHs can act as chemical stressors on phytoplankton community structure and also stimulate the synthesis of cyanotoxins by cyanobacteria. Individual exposure to GBH Faena (1.02–2.70 mg l^{-1}) resulted in reduced growth rates of the microalgae studied (*Ankistrodesmus falcatus, C. vulgaris, P. subcapitata*, and *Scenedesmus incrassatulus*), but stimulated the proliferation of the toxigenic cyanobacteria *Microcystis aeruginosa* [117]. The simultaneous presence of GLY and cyanobacteria increased stress to the microalgae. In addition, impairments in growth rate, macromolecule content, and population dynamics were observed, resulting in increased levels of catalase and glutathione peroxidase due to oxidative stress (≥ 0.74 mg l^{-1}) [117]. Additionally, changes in the external morphology and ultrastructure of microalgae were also demonstrated (e.g., loss of cell wall integrity and typical cell form, differences in starch and polyphosphate granules) [118]. Moreover, the presence of *M. aeruginosa* increased the damage observed during exposure to GBH [118]. Species-specific and dose-dependent stimulatory effect of GLY were found in several freshwater cyanobacteria species [119]. A strong correlation between reduced phosphonate levels and algal growth was demonstrated. Moreover, the uptake of phosphate was strongly dependent on the GLY concentration [119]. A concentration-dependent decrease in growth and chlorophyll-a content was observed in GLY-exposed *M. aeruginosa* cells (1–10 mg l^{-1}). Furthermore, increased malondialdehyde levels and antioxidant enzymatic activities (SOD, catalase, peroxidase) were observed (1–2 mg l^{-1}). According to the further results of the study, GLY induced apoptosis in the treated cells and triggered the release of cyanotoxin in *M. aeruginosa* [120]. After exposure to GLY (6.09 and 0.9 mg l^{-1}), a concentration-dependent growth inhibition was observed in the dinoflagellate *Prorocentrum donghaiense.* Moreover, *P. donghaiense* was unable to utilize GLY as a phosphorus source [94]. In an 8-day microcosm study, GLY led to a drastic decrease in the abundance of phycocyanin-rich picocyanobacterial by 85% [121]. Exposure to various GBHs also resulted in reduced abundance of phycocyanin-rich picocyanobacterial [122]. The abundance of phytoplankton was not affected by exposure to GLY-IPA, while increased net total abundance was observed after the exposure to GBHs (Glyphosate II Atanor and Roundup Max) [122].

Under field conditions, a decrease in chl-a was observed in the collected biofilm samples at all GLY concentrations tested (0.25–2.54 g l^{-1}). Furthermore, a dose-dependent decrease in biomass and gross primary production of autotrophs in biofilms was observed [86]. A slight decrease in algal biomass was observed after treatments with both pure GLY (100 µg l^{-1}) and a GBH

Table 2 Effects of glyphosate, its derivatives, co-formulants, and/or its formulated herbicide products on algae species reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
S. capricornutum	Roundup	4.7–60 mg l^{-1}	96 h[a]	Growth inhibition	96-h EC_{50}[b] = 15.60 mg l-1, damaged ultrastructure of cells	[78]
S. vacuolatus	Glifosato Atanor with 2.5% of the surfactant (alkyl aryl polyglycol ether)	0–8 mg AI[c] l^{-1}	96 h	Growth inhibition, metabolic and morphological endpoint	96-h EC_{50} = 4.9 mg l^{-1} metabolic and morphology alterations (≥ 4 mg l^{-1}), oxidative damage (≥ 6 mg l^{-1})	[115]
Phytoplankton community	Factor 540R	1–1000 µg l^{-1}	96 h	Diversity, physiological and biochemical parameters	reduced diversity (≥ 5 µg l^{-1}), altered pigment content (≥ 1 µg l^{-1}) and biochemical parameters (≥ 500 µg l^{-1})	[106]
Algal and cyanobacterial species	Factor 540R	10–1000 µg l^{-1}	48 h	Growth, photosynthetic parameters	48-h EC_{50} = 406–724 µg l^{-1}, altered photosynthetic response (≥ 10 µg l^{-1})	[107]
Marine phytoplankton	GLY[d]	6 and 60 mg l^{-1}	9–16 d[e]	Growth	Concentration- and species-dependent effects	[108]
Cyanobacteria species	pesticide adjuvants	screening tests	96 h	Growth	Differences in toxicity and sensitivity	[109]
Microalgae	technical-grade GLY acid, GLY-IPA[f], Roundup, POEA[g]	screening tests	96 h	Growth	POEA > Roundup > GLY acid > GLY-IPA	[30]
Algae communities	Roundup PowerFlex	0–4 mg a.e.[h] GLY l^{-1}	24 d	Diversity, density	Decreased diversity (4 mg a.e. GLY l^{-1}), no effects on density	[115]
C. vulgaris	GLY, AMPA[i]	0.05–50 mg l^{-1} individually and in combination	7 d	Growth, pigment content, antioxidant activity	Promoted growth (≤ 0.5 mg l^{-1}), growth inhibition (≥ 5 mg l^{-1}), AMPA had inhibitory effects only in the presence of GLY (≥ 5 mg l^{-1} GLY and AMPA), altered level of chl-a[j] and chl-b[k], increased antioxidant activity	[116]
Cyanobacteria, *Chlorophycean* microalgae	Faena	1–100 mg l^{-1}	96 h	Growth inhibition, macromolecules, antioxidant enzymes	IC_{50}[l] = 1.022–2.702 mg l^{-1}, effects on macromolecules and antioxidant enzymes (≥ 0.74 mg l^{-1})	[117]
Cyanobacteria, *Chlorophycean* microalgae	Faena	0.2–2.7 mg l^{-1}	96 h	Morphology and ultrastructure of microalgae	Altered external morphology and ultrastructure of microalgae, the presence of cyanobacteria represented an additional stress factor	[118]
Cyanobacterial strains	GLY	8.5–33.8 mg l^{-1}	15 d	Growth, levels of phosphate and phosphonate	Species-specific and dose-dependent stimulatory effects, decrease of phosphonate levels, concentration dependent phosphate uptake	[119]

Table 2 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
M. aeruginosa	GLY	1–10 mg l^{-1}	9 d, enzyme activity assays: 24 and 48 h	Growth, chl-a content, antioxidant activity, cell apoptosis	Reduced growth and chl-a content, increased antioxidant activity (1–2 mg l^{-1}), induced apoptosis	[120]
P. donghaiense	GLY	6.09 and 0.9 mg l^{-1}	10 d	Growth	Concentration-dependent growth inhibition	[94]
Picoplankton	GLY-IPA	4 mg l^{-1}	8 d	Chl-a, abundance	No effects on chl-a, decreased abundance of phycocyanin-rich picocyanobacteria	[121]
Picoplankton	GLY-IPA, GBHs	0.1 mg a.e. GLY l^{-1}	8 d	Chl-a, abundance	Substance-specific effects on abundance	[122]
Biofilm communities	GLY-IPA	0.25–2.54 g l^{-1}	22 d	Biofilm function and biomass	Reduced primer production (≥ 1.52 g l^{-1}), reduced chl-a	[86]
Freshwater biofilm communities	GLY-IPA, Roundup Classic	100 µg AI l^{-1}	35 d	Biomass and composition of algal communities	Initial decrease of biomass, compositional alteration	[87]
Marine microphytobenthos	Roundup	42–8500 mg l^{-1}	14 d	Microscopic analysis	Decreased abundance, compositional alteration	[123]
C. vulgaris	pure GLY, GLY-IPA, IPA, Roundup	GLY (0.51–1690.7 mg l^{-1}), GLY-IPA (0.68–2282 mg l^{-1}), IPA (0.17–591.1 mg l^{-1}), Roundup (2.23–7427.1 mg l^{-1})	21 d	Growth	Concentration-dependent and substance-specific effects	[124]
Periphyton community	Roundup	8 mg AI l^{-1}	42 d	Qualitative and quantitative analysis	Long term effects, altered abundance and composition	[125]
Freshwater periphyton	technical grade GLY acid, GBH[m]s (Glifosato II Atanor, Roundup Max)	3 mg AI l^{-1}	9 d	Pigments concentration, dry weight, algal density	Altered abundances, higher toxicity of GBHs	[126]
M. aeruginosa	GLY, Roundup	0.06–29.6 µg l^{-1}	21 d	Cell number, chl-a, photosynthesis alkaline phosphatase activity	Increased cell number and chl-a, inhibition (> 5.92 µg l^{-1}), increased photosynthesis by GLY, concentration-dependent decrease of APA activity	[127]
Phytoplankton community	GLY	0.0002 g l^{-1}	48 h	Abundance, growth	Structural and compositional effects	[128]
Freshwater periphyton	GLY IPA	0.4 or 4 mg l^{-1}	21 d	Dry weight, chl-a, periphyton abundance, recovery ability	Higher dry weight and periphyton abundance (4 mg l^{-1}), increased chl-a content (0.4–4 mg l^{-1}), recovery potential affected by GLY	[130]
Freshwater phytoplankton and periphyton communities	GLY-IPA, GIifosato Atanor	Phytoplankton: GLY 0.3, 3 and 6 mg l^{-1}; periphyton: 3 mg AI l^{-1}	7 d	Species composition, abundance and chl-a	Periphyton showed more resistance	[131]

Table 2 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
Benthic diatoms	GLY	20–500 µg l^{-1}	48 h	Microscopic analysis	Differences in sensitivity	[132]
Biofilm communities	GLY	10 µg l^{-1}	21 d	Pigment content, protein and polysaccharide content, esterase activity	Inhibited growth of autotrophic community, no effects on pigment and polysaccharide contents or enzyme activity	[133]
Biofilm communities	GLY-IPA, AMPA	GLY: 0.01–1000 mg l^{-1}, AMPA: 0.01–500 mg l^{-1}	6 h	Composition of diatom community, photosynthetic parameters, chlorophyll a content, antioxidant enzyme activities	Differences in the composition of diatom community, reduced pigment content, photosynthetic efficiency and capacity (GLY), no observed toxicity for AMPA, no effects on antioxidant enzymes	[134]
Pico- and phytoplankton	Technical-grade GLY, Roundup Max	6 mg l^{-1} (AI equivalent)	34 d	Abundance	Increased cell count	[135]
Phytoplankton, periphyton	Technical-grade GLY, Roundup Max, Glifosato Atanor	6 mg l^{-1} (AI equivalent)	14 d	Abundance and evenness of phytoplankton community, periphyton chl-a	Increased abundance of phytoplankton (GLY, Glifosato Atanor), inhibited growth of *Microcystis* spp. (Roundup Max), altered evenness, synergistic increase in periphytic chl-a	[136]
Freshwater microalgae	GLY	1.69*10^{7}–5.07 g l^{-1}	80 min	Chl-a fluorescence, cell viability	Dose-dependent effect on maximum quantum yield of $PSII^{n}$ (< 0.17 mg l^{-1})	[140]
N. microcarpa var. *wrightii*	Technical-grade GLY, Roundup, AMPA	GLY, Roundup: 0.28, 3.5, 6 mg l^{-1}; AMPA: 0.03 mg l^{-1}	7 d	Photosynthetic rate, dark respiration rate, chl-a	Higher toxicity of Roundup, stimulatory effect of AMPA	[141]
S. capricornutum	POEA	Screening tests	96 h	Growth inhibition	96-h EC_{50} = 4.1–4.9 mg l^{-1}	[104]
R. subcapitata, C. vulgaris, Oophila sp	MON 0818	Screening tests	96 h	Growth inhibition	96-h EC_{50} = 0.21–1.61 mg l^{-1}	[143]
R. subcapitata	Alkyl polyglycoside	Screening tests	72 h	Growth inhibition	Negligible aquatic toxicity	[144]
S. capricornutum	Alkyl polyglycoside	Screening tests	72 h	Growth inhibition	Variation in toxicity	[145]

[a] Day, [b]50% effective concentration, [c]active ingredient, [d]glyphosate, [e]hour, [f]GLY-isopropylammonium salt, [g]a mixture of polyethoxylated tallow amines, [h]acid equivalent, [i]aminomethylphosphonic acid, [j]chlorophyll-a, [k]chlorophyll-b, [l]half-maximal inhibitory concentration, [m]GLY-based herbicides, [n]photosystem II

(Roundup Classic) at the same GLY equivalent concentration in freshwater biofilms grown under natural conditions in Lake Balaton (Hungary) compared to the control [87]. In biofilms grown in the River Danube (Hungary), GLY (100 µg l^{-1}) initially led to a decrease in algal biomass, followed by an increase and a realignment of algal species in the biofilms. GLY-sensitive species were replaced by more tolerant ones (e.g., filamentous green species of algae), leading to a temporary decrease in biomass through various selection processes [87]. Treatment with Roundup Classic (100 µg AI l^{-1}) after 2 weeks also resulted in a decreased algal biomass in biofilms from Lake Balaton and the River Danube, with POEA increasing the toxicity of the GBH [87].

Similar selection processes have been found in natural communities of marine microphytobenthos following treatment with a Roundup GBH [123]. Several studies using standard algal growth inhibition assays [124] and community-level biofilm studies [125, 126] have demonstrated the increased combined toxicity of GLY and the additives in GBHs. At lower concentrations (0.06–29.6 µg l^{-1}), GLY can serve as a source of nutrients and phosphorus for algae species [125, 127]. In addition, GLY can also trigger pathways for protein and metabolite synthesis [108, 128], which can lead to increased biomass growth. The effects of GLY on algal communities in biofilms are highly site-specific and are greatly influenced by the specific environmental characteristics of natural aquatic habitats (e.g., dissolved oxygen content, pH), in addition to various climatic and weather conditions in different years [4, 129]. Most of the effects of GLY (0.4 mg l^{-1}) on freshwater periphyton were reversible after a recovery time of 7 days. In contrast, the higher tested concentration tested (4 mg l^{-1}) caused irreversible changes in the exposed periphyton community based on the applied recovery time of 21 days [130]. Exposure to GLY and GBHs at a much higher concentration (3 mg l^{-1}) increased the proportion of blue-green algae, while the ratio of green algae and diatoms in freshwater periphyton decreased [126]. Furthermore, the periphyton community proved to be much more tolerant to the effects of GLY compared to phytoplankton [131]. The effects of GLY on the composition of benthic diatom communities have also been demonstrated [132]. Furthermore, a higher combined toxicity of GBH formulations (such as Glifosato II Atanor, Roundup Max) was observed compared to the toxicity of technical grade GLY alone [126]. At a lower GLY concentration (10 µg l^{-1}), inhibited growth of the autotrophic community was observed in the exposed natural freshwater biofilm communities. However, no effects on pigment and polysaccharide content or esterase enzyme activity were observed [133]. Additionally, in freshwater biofilms exposed to GLY, even at very low concentration (0.01 mg l^{-1} GLY-IPA), decreased chlorohyll-a content, photosynthetic efficiency and capacity, and changes in diatom community composition [134]. Although, the toxicity of AMPA and the effects on the activity of antioxidant enzymes were not observed after either GLY or AMPA exposure [134].

The combination of technical-grade GLY or Roundup Max GBH and the presence of the invasive mussel *Limnoperna fortunei* resulted in antagonistic effects on phytoplankton [135]. The higher level of available nutrients provided by GLY was offset by the filtering activity of mussels and dramatic reductions in pico- and phytoplankton due to mussel grazing [135]. In another study, increased phytoplankton abundance was observed especially for *Microcystis* species (up to 289% and 639%) after exposure to GLY and a GBH Glifosato Atanor (6 mg AI l^{-1}), respectively. In contrast, the growth of *Microcystis* species was limited after treatment with Roundup Max [136]. The evenness of the phytoplankton community was also decreased in the exposed groups. However, the presence of *L. fortunei* significantly increased the evenness of the communities exposed to GLY or GBHs [136]. In addition to herbicides that directly inhibit photosynthesis (e.g., atrazine), other pesticide AIs such as GLY can also affect photosynthetic and respiratory processes through their effects on various metabolic pathways [127, 129]. The adverse effects of GLY on photosynthetic processes can be mainly explained by the direct or indirect inhibition of plastoquinone biosynthesis; quinone compounds are found in chloroplasts, which are crucial electron transport molecules in the light reaction of photosynthesis [137, 138]. Moreover, the decreased chlorophyll concentration [139] can directly affect the rate of electron transport in the chloroplast [129]. After GLY exposure, reactive oxygen species (ROS) generated in mitochondria can also impact photosynthesis by inhibition of the respiratory electron transport chain. Free radicals leave mitochondria and enter the chloroplast, where they cause oxidative damage to the photosynthetic apparatus and decrease photosynthesis activity [139]. The phytotoxic effects of GLY on photosynthesis activity in algae have been observed in several species of green algae and diatoms, resulting in damage to the photochemical efficiency of the PS II photochemical system [140]. In studies testing the effects of a Roundup GBH (0.28–6 mg l^{-1}, the phytotoxicity of GLY on cyanobacterial and green algal species (*M. aeruginosa, Nitella microcarpa* var. wrightii) was enhanced by the presence of POEA [141] although increased cell density, chl-a content, photosynthetic activity was also observed on algae species at lower concentrations [127], indicating a possible hormetic response that has enhanced stress effects on the plant organism [142].

The effects of GBH co-formulants have been investigated in several studies. The 72-h EC_{50} values for POEA in *P. subcapitata* ranged from 0.2 to 4.9 mg l^{-1} [30, 104, 143]. In contrast, the toxicity of alkyl polyglucosides (APGs) (C_{12-14}) was significantly higher (72-h EC_{50} = 11–46 mg l^{-1}). Significantly higher 72-h EC_{50} values of 1113–1543 mg l^{-1} were observed for APGs with shorter carbon chains (C_{8-10}) [144, 145], indicating correlation between alkyl chain length and increased toxicity.

Effects on aquatic plants

The aquatic macrophyte community serves as a microhabitat for planktonic and periphytic communities, as well as a food source for herbivorous organisms [146, 147]. Thus, observations that GLY can exert numerous detrimental effects on the aquatic macrophyte community leading to damage in food chain networks, is a serious aquatic ecotoxicological concern. The main results of ecotoxicological testing on aquatic plants are summarized in Table 3. In algal and duckweed growth inhibition tests, the inhibitory effect of AMPA on *D. subspicatus* growth was 1.5-times weaker than for a Roundup GBH. The GBH caused 100% growth inhibition (1.15 mg l^{-1}) in the common duckweed (*Lemna minor*), even at much lower concentrations compared to the ready-to-use concentration (18.38 mg l^{-1}). AMPA proved to be much less toxic [148]. Furthermore, increased ascorbate peroxidase activity and polyamine levels were observed in *L. minor* tissues after exposure to a GBH (Roundup Ultra 360 SL), although a concentration-dependent reduction was detected in the pigment content and biomass of duckweeds (≥ 360.5 mg l^{-1} AI) [149]. Additionally, the accumulation of GLY in tissues of *L. minor* exposed to 0.68 mg l^{-1} GLY-IPA, resulted in decreased growth, yield and photochemical activity of the PS II photochemical system. Moreover, inhibition of chl-a, -b, and carotenoid synthesis was also detected, while the peroxidase and catalase activities were increased at 1.6–4.56 mg l^{-1} GLY-IPA [150]. However, the inhibitory effects of a GBH Taifun Forte were found to be temperature-dependent on *L. minor* [151]. The inhibitory effect of AMPA was also demonstrated on the growth of *L. minor* exposed to AMPA (≥ 35 µg l^{-1}) [152]. In addition, a reduced chlorophyll content (30–50 µg l^{-1}) and an altered chlorophyll and amino acid metabolism were detected [152].

In *Salvinia molesta* exposed to GLY and its metabolite (≥ 40 µg l^{-1} GLY, ≥ 10 µg l^{-1} AMPA), reduced photosynthetic rates and pigment contents were observed [153]. In contrast, malondialdehyde levels and enzyme activities (catalase, ascorbate-peroxidase) were increased after GLY and AMPA exposure. In combination, the toxic effects of AMPA and GLY were enhanced. Additionally, the high removal efficiency of *S. molesta* was also demonstrated for GLY and AMPA (up to 74.2% and 71.3%, respectively) [153]. GLY (≥ 0.05 mg l^{-1}) caused growth inhibition in the submerged macrophyte *Vallisneria natans*, while the growth of *Acorus calamus* was impaired at the higher GLY concentrations tested (≥ 5 mg l^{-1}) [116]. Exposure to AMPA caused growth inhibition and increased malonaldehyde levels only at the highest concentration tested (≥ 50 mg l^{-1}). Compared to *A. calamus*, *V. natans* was more sensitive to AMPA-induced oxidative damage [116]. The combined effects of GLY and AMPA were concentration dependent and species-specific on plant growth and oxidative stress parameters [116]. In the aquatic macrophyte *Egeria densa*, decreased photosynthetic rates and chl-a content were observed after exposure to a Roundup GBH (0.28–6 mg l^{-1}) and AMPA (0.03 mg l^{-1}), while dark respiration rates increased after exposure [154].

Effects on aquatic invertebrates

The main effects of GLY and GBHs on aquatic invertebrates are presented below according to the classification of animals based on phylogenetic systematics [155]. Thus, we start with hydra, arthropods and rotifers (including zooplankton species, crabs and insects), followed by aquatic snails and mussels belonging to the phylum of mollusks, and finally with the other specialized species such as trematodes and *Echinodermata.*

Effects on hydra, arthropods and rotifers

Cnidarian species, including *Hydra viridissima*, are increasingly used as sensitive test organisms in ecotoxicological studies due to their small body size, simple anatomy, and ease of culture maintenance [156–158]. Morphological alterations were detected in *H. viridissima* exposed to GLY and the GBH Roundup Ready at a concentration of 5.2 mg l^{-1} (AI equivalent) [159]. After exposure, a high recovery capacity was observed in hydras exposed to GLY (95%). In contrast, no recovery of hydras was observed after GBH treatment [159]. Adverse effects on reproduction were indicated also after GBH exposure [159].

Zooplankton in aquatic ecosystems includes planktonic crustaceans and rotifers. This subchapter also examines scientific results for crustaceans and insects whose life cycle can be linked to aquatic environments (Table 4). Planktonic crustaceans, such as species of the genus *Daphnia*, which belong to the filter-feeding organisms, play a crucial role in aquatic ecosystems and food webs. Furthermore, due to their sensitivity to changes in water quality, daphnids are an excellent indicator species in aquatic ecotoxicology tests [160]. However, significant differences in the sensitivity of different crustaceans are occasionally observed. These differences in sensitivity

Table 3 Effects of glyphosate, its derivatives, and/or its formulated herbicide products on aquatic plants reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
Lemna minor	Roundup classic Pro, AMPA[a]	GBH[b]: 0.072–18.38 mg l^{-1}, AMPA: 0.011–2.85 mg l^{-1}	7 d[c]	Growth	100% growth inhibition (1.15 mg l^{-1} Roundup, 712.9 mg l^{-1} AMPA)	[148]
L. minor	Roundup Ultra 360 SL	360.5, 721.1, and 7206 mg l^{-1} GLY-IPA[d]	48 h[e]–3 w[f]	Growth, pigment and polyamine content, enzyme activities	Reduced pigment content and biomass (≥ 360.5 mg l^{-1}), time-dependent effects on enzyme activities and polyamine content	[149]
L. minor	GLY-IPA	0–9.13 mg l^{-1}	7 d	Growth, enzyme activities, pigment content	7-d EC_{50}[g] = 1.51 mg l^{-1}, morphological and biochemical changes	[150]
L. minor	Taifun Forte	25–400 µg l^{-1}	7 d	Growth, peroxidase activity, total protein content	Temperature-dependent growth inhibition, decreased peroxidase activity (10–30 °C)	[151]
L. minor	AMPA	5–10^5 µg l^{-1}	14 d	Growth, chlorophyll content, amino acid metabolism	Reduced growth rate (≥ 35 µg l^{-1}), reduced chlorophyll content (30–50 µg l^{-1}), altered chlorophyll and amino acid metabolism	[152]
S. molesta	GLY, AMPA	GLY: 20–100 µg l^{-1}, AMPA: 10–50 µg l^{-1}	7 d	Pigment content, photosynthetic rate, remediation potential, oxidative stress markers	Reduced photosynthetic rate and pigment content (≥ 40 µg l^{-1} GLY, ≥ 10 µg l^{-1} AMPA), increased malondialdehyde level (≥ 40 µg l^{-1} GLY, ≥ 10 µg l^{-1} AMPA) and enzyme activities (catalase, ascorbate-peroxidase) (≥ 20 µg l^{-1} GLY, ≥ 10 µg l^{-1} AMPA), removal of GLY (74.15%) and AMPA (71.34%)	[153]
V. natans, A. calamus	GLY, AMPA	0.05–50 mg l^{-1} individually and in combination	7 d	Growth, pigment content, antioxidant activity	Substance and species-specific growth inhibition, reduced chl-a[h] content), increased malondialdehyde level and SOD[i] activity	[116]
E. densa	Roundup, AMPA	Roundup: 0.28, 3.5, 6 mg l^{-1}; AMPA: 0.03 mg l^{-1}	7 d	Photosynthetic parameters, chlorophyll content	Decreased photosynthetic rates and chl-a, increased dark respiration rates (0.03 mg l^{-1} AMPA, ≥ 3.5 mg l^{-1})	[154]

[a] aminomethylphosphonic acid, [b]GLY-based herbicides, [c]day, [d]GLY-isopropylammonium salt, [e]hour, [f]week, [g]50% effective concentration, [h]chlorophyll-a, [i]superoxide dismutase

Table 4 Effects of glyphosate, its derivatives, co-formulants, and/or its formulated herbicide products on hydra, aquatic arthropods and rotifers reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
H. viridissima	GLY[a], Roundup Ready	5.2 mg l^{-1} (AI[b] equivalent)	24–96 h[c]	Mortality, morphology, feeding, reproduction, regeneration	No effects on mortality, morphological alterations, complete regeneration in 95% (GLY), no complete regeneration and reduced reproduction (GBH[d]) decreased feeding rates	[159]
D. magna, C. dubia	GBHs (Eskoba, Panzer Gold, Roundup Ultramax, Sulfosato Touchdown	0.15–40 mg a.e.[e] GLY l^{-1}	Acute test: 48 h, recovery test: 15 d[f]	Acute toxicity, recovery—survival	*C. dubia*—higher sensitivity, different neonatal mortality rates	[79]
V. fischeri, D. magna	GLY-IPA[g], GBHs (Roundup Max, Roundup Quick)	Stock solutions: GLY-IPA and Roundup Max: 6.8 g a.e. GLY l^{-1}; Roundup Quick: 7.2 g a.e. GLY l^{-1}	*V. fischeri*: 30 min, *D. magna:* 48 h	EC_{50}[h]	Significant differences in EC_{50} values and the sensitivity	[100]
D. magna, L. quadridentata	GLY, Faena	0.001–1000 mg l^{-1}	48 h	LC_{50}[i], EC_{50}, enzymatic activities	Higher toxicity of GBH	[168]
D. magna, D. spinulata	Ron-do	18–250 mg AI l^{-1}	48 h	EC_{50}	48-h EC_{50} = 61.72–66.18 mg l^{-1}	[169]
D. magna	GBHs	Screening tests	120 h	Acute toxicity	EC_{50} = 1.75 – 6.75 mg l^{-1}	[170]
D. magna	GLY, Spasor	GLY: up to 2000 mg l^{-1}, GBH: 265–355 mg l^{-1}	48 h	EC_{50}	48-h EC_{50}GBH = 299–315 mg l^{-1}, 48-h EC_{50}GLY = > 2000 mg l^{-1}	[171]
D. magna	GLY, Roundup	GBH: 5.1–15.2 mg l^{-1}; GLY: 49.8–981.8 mg l^{-1}	48 h	EC_{50}	Higher toxicity of GBH	[172]
D. magna	GLY (binary mixture of GLY and silver nanoparticles)	Acute test: 0–120 mg l^{-1}, chronic test: 2.92–14.86 mg l^{-1}	Acute test: 48 h, chronic test: 21 d	EC_{50}	48-h EC_{50} = 88–90.41 mg l^{-1}, no clear interaction, combined chronic multigenerational effects	[173]
D. magna	GLY-IPA, Roundup	Chronic test: 0–4.05 mg l^{-1}	Acute test: 48 h, chronic test: 55 d	Survival, growth, fecundity	Lower acute and higher chronic toxicity of GBH, reduced juvenile size (≥ 0.05 mg AI l^{-1}), altered growth, fecundity (GBH 0.45 mg AI l^{-1})	[174]
D. magna	Roundup	0–100 mg AI l^{-1}	48 h	Mobility, enzyme activity	Temperature-dependent toxicity	[175]
D. magna	GLY	0–600 mg l^{-1}	24 h	Acute toxicity, gene expression	Down-regulation of the *Cyp4* gene, no effects on *Cyp314* and *vtg*	[176]

Table 4 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
D. magna, C. vicinus	Sumin atut	20–640 mg l^{-1}	48 h	Acute toxicity, morphology	More sensitive *D. magna,* altered head with in both species, body length alteration of *C. vicinus*	[177]
D. magna	Sulfosato **t**ouchdown	1–8 mg AI l^{-1}	30 d	Diversity, hatching parameters	Decreased diversity (≥ 2.7 mg l^{-1}), altered community composition, altered hatching dynamics	[179]
Zooplankton	Glifosato atanor	3.5 mg AI l^{-1}	21 d	Abundance, composition	Altered composition of zooplankton	[180]
P. similis	GLY	Acute test: 1–60 mg l^{-1}, chronic test: 1–1000 µg l^{-1}	48 h	Acute toxicity, multigenerational effects	48-h LC_{50} = 33.91 mg l^{-1}, reduced growth of F0 to F6 generations, transgenerational effects	[181]
A. franciscana	Roundup	1–100 mg l^{-1} AI	48 h	Metabolome analysis	GBH effects on important metabolites	[182]
A. salina	Roundup Weed & Grass Killer	9–288 µg AI ml^{-1}, morphological analysis: 0.72–72 µg AI ml^{-1}	24–48 h	Hatching, mortality, morphology, catalase activity	Complete inhibition of hatching (144–288 µg AI ml^{-1}), no effects on mortality (≤ 72 µg AI ml^{-1}), altered early development and increased catalase activity (≥ 0.72 µg AI ml^{-1})	[183]
D. magna	POEA formulations	0.1–10,000 µg l^{-1}	48 h	Mortality, body length	LC_{50} = 97.0–849.4 µg l^{-1}, reduced body length (≥ 500 µg l^{-1})	[184]
Zooplankton	Alkyl polyglucosides	0.01–10 mg l^{-1}	1 months	Abundance, and composition	Adverse effects (≥ 2.5 mg l^{-1})	[186]
D. magna	Alkyl polyglycoside	Screening tests	24 h	Immobility	Variation in toxicity	[145]
C. nilotica (neonates, juveniles, adults)	Roundup	Neonates: 0–8 mg l^{-1}, juveniles: 0–10 mg l^{-1}, adults: 0–40 mg l^{-1}	96 h	LC_{50}, swimming behavior	LC_{50} = 2.5–25.3 mg AI l^{-1}, behavioral irregularities	[187]
N. granulata	GLY	0.02, 0.2 and 1 mg l^{-1}	72 h	Somatic and ovarian growth	Decreased body weight gain, no effects on GSI and HSI, a higher rate of reabsorbed vitellogenic oocytes (1 mg l^{-1})	[188]

Table 4 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
N. granulata	Roundup Ultramax	0.01 and 0.2 mg a.e. GLY l^{-1}	3 months	Ovarian growth	Increased glycemia, no effects on GSI, higher rate of reabsorbed vitellogenic oocytes, decreased vtg content and inhibited ovarian protein synthesis (0.2 mg l^{-1})	[189]
E. sinensis	GBH	0–400 mg l^{-1}	96 h	Acute toxicity, immune response and DNA damage	LC_{50} = 97.89 mg l^{-1}, decreased phagocytic activity, THC and rate of granulocytes, varied immune-related enzyme activities	[190]
E. sinensis	Roundup original	48.95 mg l^{-1}	7 d	Testes morphology, physiology function, and spermatozoa quality	Spermatogenesis disorder, extensive apoptosis of spermatids, altered morphology and quality	[191]
C. xanthus	Roundup original	Survival test: 2–3125 mg a.e. GLY l^{-1}, life-cycle test: 0.6–18 mg a.e. GLY l^{-1}	Survival test: 48 h, life-cycle test: 28 d	Survival, growth, emergence	LC_{50} = 251.5 mg l^{-1}, reduced larval growth, affected emergence (≥ 0.49 mg l^{-1})	[192]
Macroinvertebrates	Roundup	85 µg litter bag^{-1}	30 d	Composition, abundance	No significant effects	[193]
C. pulchellum	GLY, Roundup	1 and 2 mg AI l^{-1}	7 d	Survival, behavior, physiological parameters (e.g. food intake, swimming speed)	Higher toxicity of GBH	[194]

[a] Glyphosate, [b]active ingredient, [c]hour, [d]GLY-based herbicides, [e]acid equivalent, [f]day, [g]GLY-isopropylammonium salt, [h]50% effective concentration, [i]50% lethal concentration

can be observed in taxonomically related species such as the common water flea (*Daphnia pulex* and *Ceriodaphnia dubia*) and the great water flea (*D. magna*), although they have similar feeding strategies and lifestyles [79, 161]. In ecotoxicology tests of *D. magna* with GLY, significant differences were found, with acute toxicity (EC_{50}) values ranging from 4.2 to 24 mg l^{-1} [100, 162–165] and on occasion reaching as high as 146–930 mg l^{-1} [111, 166–168].

Similarly, reported EC_{50} values for GBHs exhibit significant variability, ranging from 1.75 to 782 mg l^{-1} [168–171]. These observed differences in EC_{50} values can be explained by variations in AI content, the presence of different co-formulants in GBHs tested, differing sensitivity between *D. magna* strains and different experimental conditions such as pH, dissolved oxygen content, or temperature. Several studies have demonstrated increased toxicity of GBHs containing POEA as a co-formulant, compared to toxicity observed with GLY alone [100, 172, 173]. However, one study found slightly lower acute toxicity of a GBH compared to GLY-IPA alone [174]. The effects of Roundup on immobility and hydrolytic enzyme activities proved to be temperature-dependent based on acute toxicity testing on *D. magna* [175]. Exposure of *D. magna* to GLY resulted in down-regulation of the *Cyp4* gene (190 mg l^{-1}), while expression of *Cyp314* remained unaffected, suggesting harmful effects on steroid and fatty acid metabolism. Additionally, vitellogenin, which is responsive to the estrogenic effect, was not affected [176]. GLY and GBH formulations caused a decrease in body size and growth of *D. magna* juveniles even at the lowest tested concentrations of GLY-IPA and a Roundup GBH (0.05 mg AI l^{-1}). Moreover, additional negative impacts were detected on reproduction rates [174]. At higher concentrations (> 20 mg AI l^{-1}), GBHs impaired the survival of *D. magna* and *Cyclops vicinus*, with observed morphological alterations in both test organisms [177]. The temperature-dependent toxicity of a Roundup GBH on alkaline phosphatase activity was also demonstrated in *D. magna*. Based on the observed results, alkaline phosphatase activity whilst playing an important role in digestion, proved to be an appropriate biomarker of damage to *D. magna* [172, 178]. Multigenerational ecotoxicology tests with a binary mixture of GLY and silver nanoparticles did not clearly demonstrate interactions between these substances [173]. However, the combined chronic multigenerational effects related to reproductive parameters (e.g., delay in the age at first brood) indicated increased toxicity compared to GLY and silver nanoparticles individually [173].

When evaluating the effects of the Sulfosato Touchdown on 30 zooplankton taxa was undertaken, a reduction in species diversity was observed above 2.7 mg l^{-1} [179]. Altered diversity, including a decrease in the proportion of cladocerans and an increase in rotifers (*Bdelloidea*), was observed in all GLY treatment groups. Additionally, treatment with this herbicide exhibited a selective impact on zooplankton hatching dynamics, including timing of first hatch and frequency of hatch [179]. Indirect effects of GBH Glifosato Atanor (3.5 mg AI l^{-1}) on zooplankton were shown with the significant increase in the abundance of rotifer species *Lecane* spp. [180]. The observed effects can be explained by the improved food availability provided by the higher abundance of picocyanobacterial and bacteria after exposure [180]. Multi- and transgenerational effects of GLY have been demonstrated in the estuarine rotifer *Proales similis* after exposure to GLY even at very low concentration (1 µg l^{-1}) [181]. In another study, sublethal exposure to a Roundup GBH resulted in a dose-dependent disruption of molting and development, as well as carbohydrate and energy metabolism in a saltwater crustacean, *Artemia franciscana* [182]. A complete inhibition of hatching was observed in GBH-exposed *Artemia salina* (144–288 µg AI ml^{-1}) [183]. In addition, altered early development and increased catalase activity (≥ 0.72 µg AI ml^{-1}) were also detected. The observed effects can be associated with excessive ROS levels and indicate the possible teratogenicity of the Roundup formulation [183].

Based on the results of ecotoxicological testing of POEA, the average 96-h EC_{50} value determined for *Daphnia* species (*D. magna* and *D. pulex*) was found to range from 0.1 to 3.8 mg l^{-1} [111, 184]. When studying the effects of GBHs, POEA was identified as the most toxic component [185]. Adverse effects of non-ionic APGs were demonstrated on *D. magna* in the concentration range of 2.5–5 mg l^{-1} [186]. Additionally, increased toxicity was observed with longer alkyl chain lengths of APGs [145].

When determining the acute effects of a Roundup GBH on the shrimp *Caridina nilotica* and its three life stages (neonates, juveniles, adults), it was found that neonates were more sensitive to the effects of the GBH at a much lower concentration (average 96-h $LC_{50} = 2.5$ mg AI l^{-1}). Behavioral abnormalities, such as slow, uncoordinated and erratic movements were also observed at all life stages [187]. Adverse effects of GLY (0.02 and 1 mg l^{-1}) and a GBH (Roundup UltraMax; 0.01 and 0.2 mg AI l^{-1}), were found on body weight gain, reabsorbed vitellogenic oocytes, vitellogenin content in the ovary of an estuarine crab (*Neohelice granulata*). The inhibition of ovarian protein synthesis was detected after the exposure to the tested GBH (0.2 mg AI l^{-1}), but GSI and HIS index were not affected [188, 189]. Furthermore, the adverse effects of a GBH on immune status, spermatophore morphology, spermatogenesis and spermatozoa quality of the Chinese

mitten crab (*Eriocheir sinensis*) were demonstrated [190, 191].

Low GLY concentrations were found to cause delayed hatching of females and rapid hatching of males in exposed midge larvae (*Chironomus xanthus*), showing negative effects at environmentally relevant concentrations (0.7 mg l^{-1}) on growth and development [192]. However, the analysis of macroinvertebrates (e.g., *Chironomidae*) did not show any effects on the diversity and abundance of macroinvertebrates after exposure to the GBH Roundup [193]. The toxicity of a Roundup GBH was higher compared to the effects of the AI on the growth rate, behavior and most physiological endpoints (e.g., escape swimming speed, food intake, fat storage) of the damselfly (*Coenagrion pulchellum*). However, some negative effects (e.g., changes in survival, muscle mass, sugar and total energy content) were observed only at the higher concentrations tested (2 mg l^{-1} GLY). These results confirm the negative effects of the POEA co-formulant on mortality and fitness of *C. pulchellum* by affecting population dynamics and predation. However, based on the results obtained, the toxic effects of the Roundup cannot be completely attributed to the presence of the surfactant [194].

Effects on mussels

Low mortality, and only few toxic effects of Roundup Express GBH and POEA on juvenile oysters (*Crassostrea gigas*) were observed at subchronic exposure (35 days) at low concentrations (≥ 0.1 µg l^{-1}) based on different parameters (e.g., shell length) [195]. However, GBHs, GLY and AMPA had no effects on embryo-larval development in *C. gigas* in the concentration range of 0.1–1000 µg l^{-1} compared to controls. Above this concentration range, a concentration dependence was observed in the severity of the detected abnormalities. Metamorphosis assays showed higher toxicity for GBHs than for GLY and AMPA [196]. After the dietary exposure to a GBH (*Scenedesmus vacuolatus* green algae exposed to Glifosato Atanor at concentration of 6 mg AI l^{-1} with the addition of 2.5% alkyl aryl polyglycol ether surfactant, biochemical alterations were detected on *Limnoperna fortunei*. A significant decrease in the carboxylesterases, while increased activity of GST and alkaline phosphatase were demonstrated. Effects on several enzyme activities (e.g., catalase, AChE, and superoxide dismutase) or oxidative damage to proteins and lipids were not proved [80]. GLY impaired acetylcholinesterase (AChE) activity and hemocyte parameters in the mussel *Mytilus galloprovincialis* due to damage to important biological processes such as endoplasmic reticulum function, energy metabolism, cell signaling, and Ca^{2+} homeostasis (≥ 10 µg l^{-1}), although no effects on antioxidant enzyme activity were observed [197, 198]. At very low concentrations (0.1 µg l^{-1}), GLY and AMPA elicited cytoprotective responses in hemocytes from treated *M. galloprovincialis* [199]. These observations appear to be due to altered efflux activity of multi-xenobiotic resistance (MXR) and altered expression of the *Abcb* gene encoding an MXR-related ABC transporter P-glycoprotein. Simultaneous exposure to GLY and AMPA induced enhanced responses in addition to the decreased efflux activity with *Abcb* down-regulation (at 1 µg l^{-1} GLY/AMPA exposure) [199]. Inhibition of AChE was detected in the mussel *Perna perna* after GLY exposure (IC_{50} = 104.8 mg l^{-1}) [200]. The studied mussel appeared to be much more sensitive than zebrafish (*Danio rerio*) and the onesided livebearer (*Jenynsia multidentate*) [200]. GLY and AMPA exposures (100 µg l^{-1}) indicate changes in the physiological homeostasis of *M. galloprovincialis* with the findings suggesting that the tested compounds may damage the animal's microbiota. AMPA caused only a slight change in the microbial community of the exposed mussels, but substantial modifications were observed after exposure to GLY and the mixture of GLY and AMPA [201]. A study of another POEA surfactant, Genamin T-200, demonstrated high toxicity on *C. gigas* embryo larval development (EC_{50} = 262 µg l^{-1}) and metamorphosis (EC_{50} = 3,027 µg l^{-1}) [202]. The most important results of the aquatic ecotoxicology tests on mussels are summarized in Table 5.

Effects on aquatic snails

The acute toxicity of GLY was demonstrated in the invasive snail *Pomacea canaliculate*, but only at high concentrations (96-h LC_{50} = 175 mg l^{-1}) [203]. Long-term exposure at sublethal concentrations (20 and 120 mg l^{-1}) resulted in inhibition of food intake, changes in metabolic profile (e.g., enhanced overall metabolic rate and modified catabolism from protein to carbohydrate/lipid mode), and impaired growth performance. In addition, increased growth was observed at 2 mg l^{-1}. Cellular responses in enzyme activities indicated increased tolerance of exposed snails by their defense system against the harmful effects of oxidative stress induced by GLY [203]. After 21 days of exposure, the effects of GLY (200 µg l^{-1}) on fatty acid composition and glutathione peroxidase activity in freshwater gastropods (*Lymnaea* sp.), were strongly dependent on temperature (20 °C and 25 °C). In addition, increased glutathione-*S*-transferase (GST) activity was observed in GLY-exposed snails, indicating the essential role of GST in the detoxification processes [204]. A Roundup GBH caused changes in mortality, reproduction, and development of *Lymnaea palustris* aquatic snails while acute steroid regulatory protein levels decreased upon treatment with the GBH, as well as

Table 5 Effects of glyphosate, its derivatives, co-formulants, and/or its formulated herbicide products on mussels reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
C. gigas	Roundup express, POEA[a]	0.1, 1, 100 µg l^{-1}	35 d[b]	Mortality, length, weight, biochemical endpoints	Low mortality, few significant differences, temporal variations of biomarkers	[195]
C. gigas	GLY[c], AMPA[d], GBHs[e] (Roundup Express, Roundup Allées et Terrasses)	0.1–10,000 µg l^{-1}	metamorphosis test: 24 h[f], embriotoxicity test: 48 h	Embryo-larval development, metamorphosis	No effects on embryo-larval development up to 1000 µg l^{-1}, higher toxicity of GBHs	[196]
L. fortunei	Glifosato Atanor + surfactant Impacto	6 mg AI[g] l^{-1} + 2,5% surfactant	28 d	Total weight, shell length, biochemical parameters (e.g., enzyme activities)	Increased GST[h] and alkaline phosphatase activity, decreased carboxylesterases activity, no oxidative damage, no effects on several enzyme activities	[80]
M. galloprovincialis	GLY	10–1000 µg l^{-1}	21 d	Biomarkers of cellular and oxidative stress	Disruption of energy metabolism, Ca^{2+} homeostasis, cell signalling, endoplasmic reticulum stress response (≥ 10 µg l^{-1})	[197]
M. galloprovincialis	GLY	10–1000 µg l^{-1}	21 d	Haemocyte and haemolymph parameters, antioxidant and AChE[i] activities	Dose- and time-dependent effects on biomarkers, (≥ 10 µg l^{-1}), no effects on antioxidant enzymes	[198]
M. galloprovincialis	GLY, AMPA	0.1–1.0 µg l^{-1}	90 min	Cytoprotective and detoxification mechanisms in haemocytes, MXR[j] activity	Induced cytoprotective responses, altered MXR activity (≥ 0.1 µg l^{-1})	[199]
P. perna	GLY	12.7–2536 mg l^{-1}	5 min	Cholinesterase activity	IC_{50}[k] = 104.8–137.0 mg l^{-1}	[200]
M. galloprovincialis	GLY, AMPA	100 µg l^{-1}	21 d	Gene expression and microbiome analysis	Affected digestive gland bacterial community, changed transcriptional levels of genes involved in immune response and apoptosis regulation	[201]
C. gigas	POEA surfactant system (Genamin T-200)	acute test: 0.1–1000 µg l^{-1}, metamorphosis test: 0.1–10,000 µg l^{-1}	Acute test: 36 h, metamorphosis test: 24 h	Embryotoxicity, metamorphose	Larval development (EC_{50} = 262 µg l^{-1}), metamorphosis (EC_{50} = 3027 µg l^{-1})	[202]

[a] A mixture of polyethoxylated tallow amines, [b]day, [c]glyphosate, [d]aminomethylphosphonic acid, [e]GLY-based herbicides, [f]hour, [g]active ingredient, [h]glutathione-*S*-transferase, [i]acetylcholinesterase, [j]multixenobiotic resistance mechanism, [k]half-maximal inhibitory concentration

after chronic exposure to GLY (3.5 mg l^{-1}) and a GBH (19.5 mg l^{-1}). Furthermore, lower testosterone and higher or equal estradiol levels were observed in snails after GLY exposure of 3.5 mg l^{-1} compared to untreated controls [205].

The co-formulant POEA in surfactant MON 0818, which is added to many commercial GBH formulations, did not significantly affect the viability of eggs of the snail *Planorbella pilsbryi* up to 9.9 mg l^{-1} [206]. However, juveniles (LC_{50} = 4.0 mg l^{-1}) were more sensitive than adults (LC_{50} = 4.9–9.1 mg l^{-1}), and egg laying was inhibited by the co-formulant (EC_{50} = 0.4–2.0 mg l^{-1}). This inhibitory effect was restored in clean water after the 96-h exposure up to 4.9 mg l^{-1}. Additionally, visible damage to tentacles of adult snails was observed at concentrations ≥ 2.7 mg l^{-1} [206]. Based on the results, environmentally relevant concentrations of GLY and surfactants (e.g., MON 0818) may pose a risk to populations of aquatic snails (Table 6).

Effects on trematodes and Echinodermata

In the natural environment, the GBH Roundup may affect the transmission dynamics and development of trematodes (*Echinostoma paraensei*) whose life cycle is associated with water courses [207]. In a study of the developmental and metabolic effects of GLY, a GBH (Roundup Power 2.0), and AMPA at environmentally relevant concentrations (1–100 µg l^{-1}) on larval sea urchin (*Paracentrotus lividus*), the observed effects were highly dependent on the type and the concentration of the tested compounds according to the parameters measured [208]. In general, GLY and AMPA showed similar levels of toxicity to the sea urchin, while the GBH formulation was less toxic than the GLY [208]. The main results of ecotoxicological tests on trematode and *Echinodermata* species are also listed in Table 6.

Effects on aquatic vertebrates

Similar to the adverse effects observed in aquatic invertebrates, GLY and the various components in GBHs may also negatively impact the health of aquatic vertebrates, such as various reptiles, fish, and amphibian species. The potential routes of exposure to aquatic contaminants may be different for these species. However, the number of ecotoxicological studies examining the effects of GLY and GBHs on reptiles is small. In aquatic turtle species (*Trachemys scripta elegans* and *Mauremys leprosa*) GBH Clinic (30 mg AI l^{-1}) significantly increased catalase and superoxide dismutase (SOD) activities of the enzymes, while reduced AChE activity was observed after the 96-h exposure. Effects on lipid peroxidation were not demonstrated [209]. In *Pelodiscus sinensis* turtles exposed to GLY-isopropylammonium (0.02–20 mg l^{-1}), no effects were observed on growth or functional performance, including food intake and swimming speed, or on liver antioxidant responses (e.g., catalase and SOD enzyme activity) and gut microbial diversity [210].

However, perturbations in hepatic metabolite profiles were detected, mainly affecting amino acid metabolism in exposed animals [210]. Exposure to a Roundup GBH (11 or 21 mg l^{-1}) altered immune parameters and complement system activity, as well as decreased white blood cell numbers and negatively affected growth were indicated in the broad-snouted caiman (*Caiman latirostris*), while total protein content was increased in the exposed animals [211, 212]. The main results of ecotoxicological studies on reptiles are summarized in Table 7.

Effects on fish species

Various fish species living in different aquatic habitats are highly exposed to chemical contaminants from industry. Contact with xenobiotics (e.g., GLY) in water is unavoidable throughout all stages of development and their life cycle. Moreover, fish species can absorb and concentrate various aquatic pollutants, which can result in food safety risks for human consumers [213]. The effects on fish observed during ecotoxicological tests are summarized in Table 8. A 24-h exposure to a Roundup GBH (10 mg l^{-1}) resulted in decreased SOD and glutathione peroxidase activity, while glutathione levels and the GST activity increased in the liver of the streaked prochilod *Prochilodus lineatus*, indicating oxidative stress [214]. AChE activity was inhibited in the brain after 96 h and in muscle after 24 h of exposure. Therefore, acute exposure to the Roundup impaired antioxidant defenses, leading to the occurrence of lipid peroxidation [214]. Reduced GST levels were observed in the South American catfish (*Rhamdia quelen*) exposed to lower Roundup GBH concentrations (≥ 0.45 mg l^{-1}) [215]. During the recovery period, increased GST activity was detected as a possible compensatory response, although catalase and SOD activity decreased, indicating toxicity from the GBH. Oxidative stress was detected during Roundup exposure possibly caused by increased protein carbonyl content and lipid peroxidation (≥ 0.45 mg l^{-1}) [215]. Increased ROS levels and cell death were observed in zebrafish (*D. rerio*) larvae exposed to Roundup Flex GBH (10 µg AI ml^{-1}) for 4 h 30 min [216]. After 14 days of exposure to GLY and a Roundup GBH at relatively low concentrations (0.01, 0.5, and 10 mg a.e. GLY l^{-1}), upregulation of the antioxidant system was observed in brown trout (*Salmo trutta*). Additionally, significant changes in the expression of transcripts encoding components of the antioxidant system, a number of stress-response proteins, and pro-apoptotic signaling molecules were observed even at the lowest dose, consistent with a cellular response to

Table 6 Effects of glyphosate, co-formulants, and/or its formulated herbicide products on aquatic snails, trematodes, and *Echinodermata* reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. canaliculata	GLY[a]	acute test: 2–300 mg l^{-1}, chronic test: 0.02–20 mg l^{-1} or 0.2–120 mg l^{-1}	acute test: 96 h[b], chronic test: 21–135 d[c]	Acute toxicity, hatching rate, physiological responses, enzyme assays	LC_{50}[d] = 175 mg l^{-1}, inhibited food intake, changes in metabolic profile (20–120 mg l^{-1}), increased growth (2 mg l^{-1}), cellular responses in enzyme activities (≥ 0.2 mg l^{-1})	[203]
Lymnaea sp.	GLY	200 µg l^{-1}	21 d	Fatty acid composition, antioxidant capacity, lipid peroxidation	Increased GST[e] activity, no effects on lipid peroxidation, temperature-dependent effects on various endpoints	[204]
L palustris	GLY, roundup	3-w mesocosm: 3.5 mg l^{-1} GLY, chronic test: GLY: 3.5 mg l-1, GBH[f]: 19.5 mg l^{-1}	3 w[g], chronic test: 6 w	Mortality, development, reproduction, steroid and protein analysis	Increased fecundity, altered steroid levels, increased level of aromatase, reduced level of steroid acute regulatory protein, higher toxicity of the GBH	[205]
P. pilsbryi	Surfactant MON 0818	0–20 mg l^{-1}	Acute test: 96 h	Survival, oviposition, viability of eggs	LC_{50} adults = 4.9–9.1 mg l^{-1}, LC_{50} juveniles = 4 mg l^{-1}, affected viability of eggs (> 9.9 mg l^{-1}), concentration-dependent reduction in egg number	[206]
E. paraensei	Roundup	225, 450 and 900 mg l^{-1}	15 min–24 h	In vitro: hatching miracidia, mortality and excystment rate of metacercariae; in vivo: parasitic load and egg production	Affected hatching miracidia rate, concentration-dependent mortality and excystment rate of metacercariae, no significant difference in parasitic load and egg production	[207]
P. lividus	GLY, AMPA[h] and Roundup Power 2.0	1–100 µg l^{-1}	48 h	Larval development and growth, respiration rate	Similar toxicity of GLY and AMPA on the respiration, GBH less toxic, altered larval development (from 10 to 50 µg l^{-1})	[208]

[a] Glyphosate, [b]hour, [c]day, [d]50% lethal concentration, [e]glutathione-*S*-transferase, [f]GLY-based herbicides, [g]week, [h]aminomethylphosphonic acid

Table 7 Effects of glyphosate and/or its formulated herbicide products on reptiles reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
T. scripta elegans, Mauremys leprosa	Clinic	30 mg l^{-1}	12 and 96 ha	Enzyme activities, lipid peroxidation	Increased catalase and superoxide dismutase activity, decreased AChE[b] activity, no effects on lipid peroxidation (96 h)	[209]
P. sinensis	GLY-IPA[c]	0–20 mg l^{-1}	30 d[d]	Growth, indicators of functional performance, gut microbial diversity, liver antioxidant responses, metabolite profiles	No significant effects on growth, functional performance (e.g., food intake), gut microbiota, liver antioxidant responses (e.g., SOD[e] and CAT[f] activities), affected hepatic metabolite profiles (≥ 0.02 mg l^{-1})	[210]
C. latirostris	Roundup	11 or 21 mg l^{-1}	2 months	Immune system and growth	Decreased white blood cell numbers (21 mg l^{-1}), higher total protein concentration (11 mg l^{-1})	[211]
C. latirostris	Roundup	11 or 21 mg l^{-1}	70 d	White blood cell, complement system activity, immune response	Reduced complement system activity, suppressive effects on the immune response	[212]

[a] Hour, [b]acetylcholinesterase, [c]GLY-isopropylammonium salt, [d]day, [e]superoxide dismutase, [f]catalase

oxidative stress as the most significant mechanism of toxicity of both GLY and its Roundup formulation [84]. The effects of GLY (2.5 and 5 mg l^{-1} for 120 h) on oxidative stress enzyme activity and malondialdehyde concentration as a marker of lipid peroxidation were detected in goldfish (*Carassius auratus*) [217]. In addition, the effects on various parameters of oxidative stress and lipid peroxidation (e.g., level of thiobarbituric acid, activity of GST and SOD) were age-specific in killifish (*Cynopoecilus* sp.) exposed to Roundup Original (65–260 µg AI l^{-1}) [218].

A slight decrease in the number of erythrocytes, as well as hemoglobin and hematocrit levels were also observed compared to controls, indicating moderate anemia in the exposed goldfish [217]. In guppy (*Poecilia reticulata*) gill erythrocyte cells exposed to different concentrations of Roundup Transorb GBH (0.91–3.66 mg l^{-1}) for 24 h, a concentration-dependent increase in the number of damaged cells was observed, indicating mutagenic and genotoxic effects [219]. Genotoxic effects of another GBH (Roundup Full II—2.75 mg l^{-1}) were detected in the blood, liver and gill cells of exposed pacu fish (*Piaractus mesopotamicus*) [220]. The genotoxic potential of GLY, Roundup, and POEA was detected in blood cells of the exposed European eel (*Anguilla anguilla*) [221]. Altered hematological and biochemical parameters (e.g., decreased level of alkaline phosphatase, hemoglobin and hematocrit value, increased level of white blood cells) were also observed in *Labeo rohita* after chronic exposure to GBH Roundup (0.63–2.06 mg l^{-1}) [222].

Lower heart rates were observed in treated *D. rerio* embryos (100 and 1000 µg l^{-1} of GLY at 48 h), indicating possible cardiotoxicity [223]. Altered transcriptome profiles (30 differentially expressed genes involved in metabolic processes, oocyte maturation, and nervous system development) were also observed in these embryos at the higher GLY concentration after 96 h [223]. The cardiovascular toxicity of GLY was also demonstrated in *D. rerio* embryos exposed to 30–120 µg ml^{-1} GLY up to 72 h after fertilization [224]. Cardiac malformations, including enlarged chambers, rhythm alterations, and thinned ventricular walls, as well as a defective intersegmental vasculature indicative of damaged angiogenesis, were observed in the exposed embryos. The cardiovascular effects of GLY might be related to apoptosis, as apoptosis occurs in the cardiac and vascular regions. Additionally, altered development, hatching abnormalities, mortality, and decreased body length of exposed embryos were also observed [224]. Exposure to GLY and Roundup Original DI (250–1000 µg l^{-1}) caused decreased heart rate and decreased activity of GST and AChE in exposed *D. rerio* embryos [225]. Effects on behavior and various biochemical parameters (e.g., total antioxidant capacity, lipid peroxidation, ROS level) were not observed. A higher rate of malformations (e.g., pericardial edema, yolk sac edema, and curvature of the spine) was observed in GBH-exposed embryos [225].

When tambaqui (*Colossoma macropomum*) were exposed to Roundup GBH (10 and 15 mg AI l^{-1}), altered

Table 8 Effects of glyphosate, its derivatives, co-formulants, and/or its formulated herbicide products on fish reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. lineatus	Roundup	10 mg l^{-1}	6–96 ha	Antioxidant analysis, AChE[b]	Reduced SOD[c] and glutathione peroxidase activity, increased glutathione levels and GST[d] activity, inhibited AChE in brain (96 h) and in muscle (24–96 h)	[214]
R. quelen	Roundup	0.45 and 0.95 mg l^{-1}	8 d[e]	Oxidative stress parameters (e.g., antioxidant enzymes, GST activity)	Increased thiobarbituric acid reactive species and protein carbonyl levels (≥ 0.45 mg l^{-1}), decreased GST activity (≥ 0.45 mg l^{-1}), no effects on SOD and ascorbic acid	[215]
D. rerio	Roundup Flex	1, 5 and 10 mg AI[f] l^{-1}	4 h 30 min	Biochemical parameters (e.g., ROS[g], SOD, lipid peroxidation, apoptosis)	Increased ROS and cell death (10 mg AI l^{-1}), no effects on SOD, GSH and lipid peroxidation	[216]
S. trutta	GLY[h], roundup	0.01–10 mg a.e.[i] GLY l^{-1}	14 d	Transcripts encoding components of the antioxidant system, stress-response proteins and pro-apoptotic signaling molecules	Upregulation of the antioxidant system, significant changes in the expression of transcripts encoding components of the antioxidant system, stress-response proteins, pro-apoptotic signaling molecules	[84]
C. auratus	GLY	2.5–5 mg l^{-1}	120 h	Hematological and biochemical parameters	Decreased SOD, glutathione peroxidase (5 mg l^{-1}), and catalase (≥ 2.5 mg l^{-1}), increased malondialdehyde concentration, slightly decreased number of erythrocytes, hemoglobin and hematocrit levels	[217]
Annual killifish (*Cynopoecilus* sp.)	Roundup original	65–260 µg AI l^{-1}	7 d	Oxidative stress parameters (e.g., SOD, GST, lipid peroxidation)	Age-dependent effects on the activity of SOD, catalase and the level of thiobarbituric acid reactive species, no effects on catalase activity	[218]
P. reticulata	Roundup transorb	0.91–3.66 mg l^{-1}	24 h	Micronucleus, nuclear abnormalities	Concentration-dependent frequency of nuclear abnormalities, increased number of damaged cells	[219]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. mesopotamicus	Roundup full II	2.75–13.25 mg l^{-1}	96 h	Acute toxicity, micronucleus, nuclear abnormalities	LC_{50}[j] = 8.92 mg l^{-1}, higher frequency of micronucleus and nuclear abnormalities (2.75 mg l^{-1})	[220]
A. anguilla	GLY, Roundup Ultra, POEA[k]	GLY: 17.9–35.7 µg l^{-1}, GBH[l]: 58–116 µg l^{-1}, POEA: 9.3–18.6 µg l^{-1}	1–3 d	Genetic damage	Genotoxic potential of GLY, GBH and POEA	[221]
L. rohita	Roundup	acute test: 6–14 mg l^{-1}, chronic test: 0.63–2.06 mg l^{-1}	Acute test: 96 h, chronic test: 28 d	Haematological and biochemical parameters	96-h LC_{50} = 10.16 mg l^{-1}, reduced level of red blood cells, hemoglobin, and hematocrit value, increased level of white blood cells, altered biochemical and stress parameters (e.g., decreased activity of blood glucose, total protein, and alkaline phosphatase	[222]
D. rerio	GLY	0–1000 µg l^{-1}	96 h	Mortality, heart rate, hatching rate	No mortality, lower heart rates (100 and 1000 µg l^{-1} at 48 h), altered transcriptome profiles (1000 µg l^{-1} after 96 h)	[223]
D. rerio	GLY	30–120 mg l^{-1}	72 h	Survival and hatching rate, body lengths, embryo morphology, apoptosis, cardiovascular toxicity	Reduced survival, cardiac malformations apoptosis in the cardiac and vascular regions (≥ 60 mg l^{-1}), decreased hatching rate and body length (≥ 90 mg l^{-1}), altered caspase activity and ATP[m] level	[224]
D. rerio	GLY, roundup original DI	250–1000 µg l^{-1}	96 h	Rate of survival and hatching, heart rate, malformations, behavior, biochemical biomarkers (e.g., GST and catalase, lipid peroxidation)	Decreased survival (500 µg l^{-1} GBH), no behavioral effects, increased hatching (GBH at 48 hpf[n]), higher rate of malformations (GBH), reduced heart rate, decreased activity of GST and AChE, no effects on other biochemical parameters	[225]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
C. macropomum	Roundup	10 and 15 mg AI l^{-1}	96 h	Blood analysis, enzyme activities	Affected hematological parameters and biotransformation processes, ROS production, increased DNA damage, inhibited AChE activity	[226]
C. punctatus	Roundup	3.25–6.51 mg AI l^{-1}	35 d	DNA damage, lipid peroxidation, antioxidant enzymes activities	Concentration-dependent and tissue-specific DNA damage, increased lipid peroxidation, suppressed antioxidant enzymes	[227]
D. rerio	GLY	700 µg l^{-1}	28 d	Hepatic metabolism	Sex-specific effects on hepatic metabolism, increased stress responses	[228]
D. rerio	GLY	0.7–35 mg l^{-1}	1–1.5 h	Body length, heart rate, hormone levels, oxidative stress, inflammation reactions, apoptosis	Malformations, reduced heart rate and body length (≥ 7 mg l^{-1}), decreased T3/T4 ratio (35 mg l^{-1}), oxidative stress (≥ 7 mg l^{-1}), induced inflammatory response and apoptosis	[229]
D. rerio	GLY, AMPA, GBHs (Dominator Extra 680 SL, Fozat 480, Roundup Mega, Total	0.35–2.8 mg a.e. l^{-1}	120 hpf	Development, in vivo estrogenicity	Acute toxicity: AMPA > GLY > GBHs, sublethal abnormalities (0.35–2.8 mg l^{-1} GBHs), in vivo estrogenicity	[230]
C. carpio, in vitro: cell fish line	GLY	In vivo: 0.5 and 15 mg l^{-1}; in vitro: 0.65 and 3.25 mg l^{-1}	in vivo: 30 d; in vitro: 48 h	In vivo: histopathological analysis, biochemical analysis, swimming behavior; in vitro: cell proliferation, intracellular ROS, mitochondrial membrane potential, DNA damage	In vivo: oxidative DNA damage, liver inflammation, altered physical intestinal barrier (5–15 mg l^{-1}), inhibited AChE (15 mg l^{-1}), altered swimming behavior; in vitro: ROS production, mitochondrial dysfunction, reduced cell viability	[231]
D. rerio	GLY	1, 65, and 5000 µg l^{-1}	72 h	Nuclear abnormalities and micronuclei	Increased frequency of nuclear morphological abnormalities and micronuclei formation	[232]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
D. rerio, C. carpio	GLY	0.005–50 mg l^{-1}	120 h	Mortality, hatching rate, developmental disorders	Highest cumulative mortality at 50 mg l^{-1}, reduced hatching rate in *C. carpio* (especially at 50 mg l^{-1}), hatching stimulation in *D. rerio* (96 hpf), concentration-dependent malformations	[81]
D. rerio	GLY	65 µg l^{-1}	15 d	Morphological ultrastructural, biochemical parameters	No observable changes in general morphology, but altered ultrastructure of ovaries, increased oocyte diameter, higher expression of SF-1	[233]
D. rerio	GLY, AMPA	1–700 ng ml l^{-1}	72 h	Morphological changes, apoptotic cells, ATPase activity	Reduced survival, hatching and deformity rate (≥ 10 ng ml l^{-1}), disturbed cardiac development and apoptosis, inhibited Na + /K + -ATPase and Ca2 + -ATPase activity (≥ 1 ng ml l^{-1})	[234]
D. rerio	GLY, Roundup	GLY: 10 mg l^{-1}, GBH: 0.01–10 mg a.e. GLY l^{-1}	21 d	Mortality, reproductive parameters (e.g., hatching)	Decreased egg production, but no effects on fertilization rate (10 mg l^{-1} GLY), increased mortality and premature hatching (10 mg l^{-1} GLY/GBH)	[235]
J. multidentata	Roundup Max	Acute test: 5–100 mg l^{-1}, chronic test: 0.5 mg l^{-1}	Acute test: 96 h, chronic test: 28 d	LC_{50}, male sexual activity, histological analysis, gill morphometric	LC_{50} = 19.02 mg l^{-1}, concentration-dependent histological changes in the gills and liver (≥ 0.5 mg l^{-1}), reduced number of copulations and mating success in males	[236]
D. rerio	GLY	5 or 10 mg l^{-1}	24 or 96 h	Sperm quality (e.g., concentration and motility)	No effects on sperm concentration, reduced motility, mitochondrial function, sperm membrane and DNA integrity (≥ 5 mg l^{-1})	[237]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
D. rerio	GBH	1–100 mg AI l^{-1}	24–96 h	Mortality, morphological abnormalities, carbonic anhydrase activity, ROS, apoptosis	Decreased survival (≥ 1 mg l^{-1}) and hatching rate (100 mg l^{-1}), malformations and decreased carbonic anhydrase activity (≥ 1 mg l^{-1}) ROS production, increased apoptosis	[238]
H. transpacificus	Roundup	0.064–640 mg AI l^{-1}	6 h	Hepatic 17β-estradiol, AChE, total glutathione	No effects on AChE, altered 17β-estradiol in males (≥ 0.078 mg l^{-1}), reduced glutathione concentration in males (0.7 mg l^{-1})	[239]
C. gariepinus	Forceup	0.02–1 mg l^{-1}	24 h–8 d	Haematological and biochemical parameters, exploratory behavior, growth performance	Decreased body weight, (≥ 0.02 mg l^{-1}), growth (≥ 0.05 mg l^{-1}) and survival (1 mg l^{-1}), altered behavioral parameters (≥ 0.02 mg l^{-1}), increased malondialdehyde (≥ 0.05 mg l^{-1}), decreased reduced glutathione and SOD activity (≥ 0.1 mg l^{-1}), hematological effects	[240]
O. mykiss	GLY, GBHs (roundup innovert and Viaglif Jardin)	1 µg l^{-1}	10 months	Survival of the eggs, hematologic, immunologic, metabolic parameters, oxidative stress	No effects on average body weight, relative fecundity, fertility, and enzyme activities, decreased level of macrophages and phagocytic activity (GBHs, two months before spawning). Lower level of tumor necrosis factor-α (1 month after spawning)	[241]
C. decemmaculatus	Roundup Max	0.2–2 mg l^{-1}	42 d	Somatic indexes (condition factor, hepato-somatic index), locomotor activity, enzymatic activities (e.g., AChE, catalase, GST)	No effects on locomotor activity, somatic indexes, AChE and catalase activity Higher GST activity in liver, reduced aspartate aminotransferase, alanine aminotransferase activity	[242]
C. decemmaculatus	GLY	1–35 mg l^{-1}	96 h	AChE activity	Inhibited AChE activity (≥ 1 mg l^{-1})	[243]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
C. carpio	Roundup	0.5–10.0 mg l^{-1}	96 h	AChE activity, oxidative stress	Inhibited AChE activity (increased level after the recovery period), increased levels of thiobarbituric acid reactive species in the brain	[244]
R. quelen	GBHs (Orium, Roundup Original and Biocarb)	2.5 and 5.0 mg AI l^{-1}	96 h	Oxidative stress indicators (e.g., TBARS[o], antioxidant enzymes), transaminases	Increased TBARS and reduced catalase activity (≥ 2.5 mg l^{-1}), significant effects on SOD and GST, moderate to severe histopathological alterations, reduced levels of transaminases (Biocarb: 2.5 mg AI l^{-1})	[245]
P. reticulata	GLY, AMPA[p]	GLY: 50–73.2 mg l^{-1}, AMPA: 86.8–180 mg l^{-1}	96 h	LC_{50}, histopathological and histomorphometric analyses	LC_{50}GLY = 68.78–70.87 mg l^{-1}, LC_{50}AMPA = 164.3–180 mg l^{-1}, sex- and tissue-specific histopathological responses in the gills and liver (GLY: 35 mg l^{-1}, AMPA: 82 mg l^{-1})	[246]
Surubim (crossbred between two Neotropical catfish species)	Roundup original	2.25–15 mg l^{-1}	96 h	Metabolic and behavioral parameters	Concentration-dependent and tissue-specific effects on metabolic parameters (e.g., glucose, lactate levels), decreased plasma cholesterol (2.25–15 mg l^{-1}), concentration-dependent behavioral effects	[247]
D. rerio	GLY, roundup	0.01–0.5 mg AI l^{-1}	96 h	Survival, morphological defects, behavioral parameters (e.g., exploratory and aggressive behavior, aversive memory)	No effects on survival, reduced body length (GBH: 0.01–0.5 mg AI l^{-1}) and altered behavior (≥ 0.01 mg AI l^{-1}) in larvae, in adults: altered exploratory (≥ 0.065 mg l^{-1}) and aggressive (≥ 0.01 mg AI l^{-1}) behavior, impaired memory	[248]
C. carpio	Aria	20.5–61.5 g l^{-1}	96 h	LC_{50}, histopathological and behavioral parameters	LC_{50} = 28.2 g l^{-1}, hyperplasia, hypertrophy, and hyperemia of the gills, significant differences in the swimming behavior	[249]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
Crossbred red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*)	GLY	25–150 mg l^{-1}	7 weeks	Growth, somatic indexes	Concentration-dependent growth inhibition, alteration of HSI[q] and GSI[r], decreased body weight and length (≥ 25 mg l^{-1})	[250]
P. reticulata	Roundup transorb	0.34–5.2 mg GLY l^{-1}	2–96 h	LC_{50}, proteomic response, histopathological effects	LC_{50} = 3.4 mg l^{-1}, time-dependent histopathological effects in the gills (1.82 mg AI l^{-1}), regressive, vascular and progressive disorders were indicated by histopathological indexes, 14 proteins (e.g., involved in metabolism and stress response) were affected	[251]
C. carpio	GLY	5 and 15 mg l^{-1}	60 d	Growth, AChE activity, behavior parameters, microbiome	Reduced weight growth (15 mg l^{-1}), affected blood–brain barrier permeability (≥ 5 mg l^{-1}), decreased AChE activity, reduced swimming speed (5 mg l^{-1}), altered microbiome	[252]
O. niloticus	GLY	2 mg l^{-1}	60 d	Biochemical parameters, transcriptome and proteome analysis	No effects on GPT[s] and GOT[t] activities, increased SOD activity and MDA[u] content, reduced GSH[v] and T-AOC[w] levels, 94 up-regulated and 131 down-regulated genes (e.g., involved in ion transport, lipid metabolism)	[253]
Novel 3D *S. salar* co-culture model (primary hepatocytes and kidney epithelial cells)	GLY	0.8–84.5 mg l^{-1}	48 h	Cell viability, metabolomic profiling	Higher cell viability in 3D cultivated hepatocytes, affected lipid metabolism, increased cholesterol level and down-regulation of clusterin (84.5 mg l^{-1})	[254]
D. rerio	GLY, AMPA	0.02–1.69 mg l^{-1} (combination 1:1)	7 d	Survival, hatching, ROS, behavioral parameters	No effects on mortality, hatch success, development, and ROS production, concentration-dependent and substance-specific effects on enzymes (e.g., SOD), hyperactivity (GLY), lack of anxiety-like behaviors	[255]

Table 8 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
O. niloticus	Roundup	0.6 mg AI l^{-1}	2–4 weeks	Oxidative stress, immunosuppression, liver and kidney dysfunction	Reduced body weight, no effects on growth, liver and kidney dysfunction, reduced level of tested proteins, oxidative stress response, antioxidant activity, lipid peroxidation, immunosuppression	[256]
D. rerio	GLY	Larvae: 0.2456 µg l^{-1} larvae/day (dietary) Adults: 1, 5, and 10 mg l^{-1}	96 h	Larvae: survival, adults: bioconcentration factors in the gills, liver, and muscle	Increased mortality, affected energy metabolism and feeding behavior of larvae, altered dynamics between zooplankton and fish larvae, bioaccumulation in zooplankton	[257]
P. lineatus	POEA	0.15–1.5 mg l^{-1}	24 h	DNA damages, biochemical and physiological parameters	Increased plasma lactate levels and decreased hepatic catalase activity, red blood cell counts and hemoglobin content, DNA damage, lipid peroxidation and hemolysis, no effects on hematocrit levels	[258]
A. anguilla	GLY, Roundup Ultra, POEA	GLY: 17.9 and 35.7 µg l^{-1}, GBH: 58 and 116 µg l^{-1}, POEA: 9.3 and 18.6 µg l^{-1}	1–3 d	DNA damage, GDI	Higher levels of DNA damage (GBH: 116 µg l^{-1}, GLY: 17.6 µg l^{-1}, POEA: 9.3–18.6 µg l^{-1}, synergistic interaction between POEA and GLY in promoting non-specific DNA damage	[221]
D. rerio	GLY, Atanor 48, AMPA, POEA	GLY, GBH, AMPA: 1.7–100 mg l^{-1}, POEA: 0.4–16 mg l^{-1}	96 h	Acute toxicity, genetic damage index	No acute toxic effects of GLY and AMPA, LC_{50}GBH = 76.50 mg l^{-1}, LC_{50}POEA = 5.49 mg l^{-1}, genotoxic potential, direct genotoxic properties of POEA	[259]

[a] Hour, [b]acetylcholinesterase, [c]superoxide dismutase, [d]glutathione-*S*-transferase, [e]day, [f]active ingredient, [g]reactive oxygen species, [h]glyphosate, [i]acid equivalent, [j]50% lethal concentration, [k]a mixture of polyethoxylated tallow amines, [l]GLY-based herbicides, [m]adenosine triphosphate, [n]h post-fertilization, [o]thiobarbituric acid reactive substances, [p]aminomethylphosphonic acid, [q]hepatosomatic index,[r]gonadosomatic index, [s]glutamic pyruvic transaminase, [t]glutamic-oxalacetic transaminase, [u]malondialdehyde, [v]glutathione, [w]total antioxidant capacity

biotransformation processes were observed in the gills [226]. In addition, ROS were produced in the liver and increased DNA damage was observed in red blood cells. Furthermore, inhibition of AChE activity was also observed in the exposed fish brain [226]. Concentration-dependent DNA damage and increased levels of ROS and lipid peroxidation were observed in the spotted snakehead (*Channa punctatus*) exposed to sublethal GLY concentrations (Roundup 3.25–6.51 mg AI l^{-1}). However, the extent of lipid peroxidation and DNA damage was higher in gills than in blood cells [227].

Sex-specific disruption of the hepatic metabolism in zebrafish (*D. rerio*) was detected after the longer-term exposure (28 days) at a lower GLY concentration (700 µg l^{-1}). In females, decreased uridine 5'-monophosphate content was observed in the pyrimidine metabolic pathway, as well as the reduction of purine intermediates was indicated. In addition, decreased aminoadipic acid in the lysine degradation pathway observed in males [228]. GLY exposure also resulted in increased stress responses in both sexes, namely an increased stress-inflammatory response in females and an impaired oxidative stress response in males [228]. Exposure to GLY (35 mg l^{-1}) caused decreased triiodothyronine (T3)/thyroxine (T4) ratios in exposed *D. rerio* embryos (120 h post-fertilization) [229]. Moreover, abnormal expression patterns of genes related to the hypothalamic-pituitary-thyroid and growth hormone/insulin-like growth factor axes were observed. As a result, developmental toxicity was demonstrated in these fish (e.g., reduced heartbeats, premature hatching, shortened body, swim bladder deficiency, pericardial and yolk sac edema) (≥7 mg l^{-1}). No oxidative stress or significant malformations were detected at the lowest concentration, but hormonal changes were observed. GLY at 7 and 35 mg l^{-1} caused accumulation of ROS in larvae [229]. In vivo, the estrogenicity of AMPA, GLY and GBHs was demonstated in an estrogen-sensitive, transgenic zebrafish line after 120 h of exposure (0.35–2.8 mg l^{-1}) [230]. The acute toxicity of AMPA was not detected, while the toxicity of GBHs was higher compared to GLY. In addition, sublethal anomalies and malformations were observed in the GBH-exposed embryos [230].

Oxidative DNA damage and production of ROS was observed in juvenile common carp (*Cyprinus carpio*) (≥5 mg l^{-1}). Liver inflammation in vivo, accompanied by oxidative damage and altered physical intestinal barrier, was observed in carp exposed to GLY concentrations (5–15 mg l^{-1}) for 30 days. Moreover, at 15 mg l^{-1} GLY, inhibition of AChE activity in the brain of the fish was observed, and decreased swimming speed and distance, as well as average acceleration were demonstrated [231]. In addition, also oxidative DNA damage, ROS production, mitochondrial dysfunction, and reduced cell viability were detected on the tested fish cell line (0.65 and 3.35 mg l^{-1}) [231]. In another study, an increased frequency of nuclear morphological abnormalities and micronuclei formation were observed in *D. rerio* exposed to GLY (1, 65, and 5000 µg l-1 for 72 h) [232].

After the common carp (*C. carpio*) and zebrafish (*D. rerio*) were exposed to GLY at various concentrations (0.005–50 mg l^{-1}) at early life stages, a delay in hatching was observed, especially at the highest concentration after 72, 96, and 120 h post fertilization. In contrast, hatching stimulation was observed in *D. rerio* embryos exposed to GLY (96 h post fertilization). Early life stages of *C. carpio* were more sensitive, with numerous malformations and delayed development compared to *D. rerio*. GLY at lower concentrations (0.005 mg l^{-1}) resulted in significant changes in both fish species, including altered mortality and occurrence of malformations, possibly reducing biodiversity [81]. Long-term exposure to low concentrations of GLY (65 µg l^{-1} for 15 days) showed adverse effects on reproduction in *D. rerio*, with a significant increase in oocyte diameter associated with the appearance of concentric membranes resembling myelin-like structures in the ultrastructure of ovaries correlating with the outer membranes of mitochondria and with yolk granules [233]. Low concentrations of GLY and AMPA (≥10 ng ml^{-1}) caused developmental toxicity in zebrafish embryos (exposure from 2 to 74 h post-fertilization for 72 h), with concentration-dependent heart rate elevation and arrhythmia observed [234]. In exposed embryos, disturbances in heart development were observed, possibly related to altered transcription levels of genes involved in development and apoptosis. Pericardial edema and bone deformities were also observed as a possible consequence of inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase after GLY and AMPA exposure (≥1 ng ml^{-1}) [234].

Reproduction of *D. rerio* was affected by 21-day exposure to GLY and GBH Roundup, while GLY (10 mg l^{-1}) caused decreased egg production in breeding colonies, although fertilization rate was not affected. Moreover, both Roundup and GLY (10 mg l^{-1}) increased mortality and premature hatching of early-stage embryos [235]. In the one-sided livebearer (*J. multidentata*) concentration-dependent histological changes were observed in the gills and liver after the exposure to Roundup (≥0.5 mg l^{-1}). In addition, the number of copulations and mating success decreased in male fish [236]. Adverse effects of GLY (5 and 10 mg l^{-1}) on sperm quality of *D. rerio* were observed after 24 and 96-h exposure, including damage to sperm membranes and DNA. Moreover, reduced mitochondrial function and sperm motility were detected, suggesting reduced fertility (≥5 mg l^{-1}) [237]. In *D. rerio* embryos exposed to GBH (1–100 mg AI l^{-1}) for 24 to 96 h, a

dose-dependent inhibition of carbonic anhydrase activity was observed, which was attributed to the production of ROS, especially in branchial regions, caused by cellular apoptosis [238].

Various types of malformations were also observed in a dose-dependent manner, including pericardial edema, spinal curvature, yolk sac edema, and body malformation (≥ 1 mg AI l^{-1}) [238]. Furthermore, a negative effect of Roundup GBH (78 µg AI l^{-1}) on the concentration of 17β-estradiol and reduced glutathione concentration was observed in the liver of male delta smelts (*Hypomesus transpacificus*) (700 µg AI l^{-1}) [239]. Decreased body weight, altered morphology (24 h post-fertilization), survival rate, growth, and behavioral parameters were demonstrated in *Clarias gariepinus* exposed to the GBH Forceup (0–1 mg l^{-1}) at different developmental stages (e.g., gametes, postfryer, juvenile) [240]. Moreover, increased levels of malondialdehyde were detected at the higher GBH concentrations indicating an oxidative stress response after GBH exposure. Decreased levels of reduced glutathione and SOD activity were found in exposed post-fingerlings and juveniles compared to controls. Histological analysis revealed necrosis in the gills, cardiac myocytes, brain, and liver of exposed fish [240].

After daily exposure of the rainbow trout (*Oncorhynchus mykiss*) to 1 µg l^{-1} GLY and GBHs (Roundup Innovert and Viaglif Jardin) for 10 months during spawning, no effects on average body weight, relative fecundity, and fertility were observed [241]. However, fish exposed to the GBH Viaglif Jardin two months before spawning showed a 70% decrease in the proportion of macrophages and a 35% decrease in phagocytic activity. One month after spawning, a lower tumor necrosis factor-α level was observed, but the difference was not significant compared to the control [241]. No effects on locomotor activity, somatic indexes, AChE and catalase activity were demonstrated in adult females of the ten spotted live-bearer (*Cnesterodon decemmaculatus*) exposed to GBH Roundup Max (0.2 and 2 mg l^{-1}) for 6 weeks. However, the activity of GST in liver, reduced aspartate aminotransferase, and alanine aminotransferase were significantly affected in the exposed fish [242]. Additionally, GLY at a concentration of 1 mg l^{-1} acted as a significant AChE inhibitor in *C. decemmaculatus* [243].

The inhibitory effect of a Roundup GBH (0.5–10.0 mg l^{-1} for 96 h) on AChE activity was also detected in the brain and muscle of exposed common carp (*C. carpio*), although AChE activity increased after the recovery period. Moreover, increased levels of thiobarbituric acid reactive species (TBARS) were measured in the brain, indicating oxidative stress [244]. Increased TBARS levels were also found in the silver catfish (*R. quelen*) exposed to different GBHs (e.g., Orium, Roundup Original, and Biocarb) at concentrations of 2.5 and 5.0 mg l^{-1} for 96 h. However, the amount of catalase produced in the liver decreased in all treatments [245]. A sex- and tissue-specific histopathological response was observed in the gills and liver of guppies (*P. reticulata*) exposed to GLY (35 mg l^{-1}) and AMPA (82 mg l^{-1}) for 96 h [246]. Male fish showed more frequent hepatic inflammatory changes and a higher increase in the area of hepatocyte vacuoles compared to female fish exposed to GLY and its metabolite. Male guppies exhibited higher sensitivity than females, particularly in the presence of AMPA [246]. In the hybrid fish surubim (crossbred between two Neotropical catfish species, pintado, *Pseudoplatystoma corruscans* × cachara, *P. reticulatum*), exposure to the GBH Roundup Original (≥ 2.25 mg l^{-1}) for 96 h resulted in reduced plasma glucose levels but increased levels in the liver, while lactate levels increased in both plasma and liver and decreased in muscles [247]. In addition to the concentration-dependent and tissue-specific effects of the GBH, plasma cholesterol concentration decreased at all concentrations tested. Moreover, altered behavioral parameters such as ventilatory frequency and swimming activity were observed at higher concentration (≥ 2.25 mg l^{-1}) [247].

Exposure to GLY and a Roundup GBH (0.01–0.5 mg AI l^{-1}), resulted in altered morphology and behavior of *D. rerio* even at the lowest concentration tested after a 96-h exposure [248]. Adult fish showed reduced exploratory (≥ 0.065 mg AI l^{-1}) and aggressive behavior (≥ 0.01 mg AI l^{-1}). In the exposed larvae, altered exploratory and aversive behavior were also observed (≥ 0.01 mg AI l^{-1}). Impaired memory was observed in adult fish exposed to Roundup (0.5 mg AI l^{-1}), and exposure to GLY (0.5 mg l^{-1}) resulted in reduced ocular distance in larvae [248]. In *C. carpio*, significant differences were found in the swimming behavior of fish treated with GLY (50, 100, and 150 mg l^{-1}), along with additional clinical signs such as increased movement of the operculum and darkening of the skin. Hyperplasia, hypertrophy, and hyperemia of the gills were also observed [249].

After a 7-week exposure to a range of GLY concentration (25–150 mg l^{-1}), crossbred red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) showed differences in growth pattern, hepato-somatic index, and gonado-somatic index with decreased body weight even at the lowest concentration tested [250]. Time-dependent histopathological effects were observed in the gills of guppies exposed to a GBH (1.82 mg AI l^{-1}), with various epithelial and muscle cell types showing progressive, regressive, and vascular disorders [251]. In *C. carpio* exposed to GLY (5 and 15 mg l^{-1}) for 60 days, a statistically significant decrease mRNA expression of tight-junction genes and inhibition of AChE activity was

observed at the higher concentration [252]. In addition, the combination of GLY (15 mg l^{-1}) and polyethylene microplastics (4.5 mg l^{-1}) led to the inhibition of free-swimming behavior of carp [252]. Exposure of tilapia (*O. niloticus*) to GLY (2 mg l^{-1}) resulted in dramatic changes in gene expressions, with 94 up-regulated and 131 down-regulated genes [253]. Long-term effects of GLY on 21 proteins related to liver metabolic function were also observed, indicating a redox imbalance and dysregulation of metabolism in exposed fish [253]. In an in vitro 3D hepatocyte-kidney co-culture model, GLY (84.5 mg l^{-1}) affected lipid metabolism in Atlantic salmon (*Salmo salar*) hepatocytes and kidney cells after a 48-h exposure, leading to an increased cholesterol level and down-regulation of clusterin, which may affect the stability of the kidney cell membrane [254].

Mortality, hatch success, development, and ROS production were not affected by GLY and AMPA (neither individually nor in combination) in exposed *D. rerio* embryos l-1arvae 7 days after fertilization compared to controls [255]. However, the activity of tested enzymes (e.g., SOD) was altered in a concentration- and a compound-specific manner. Hyperactivity was detected in fish treated with GLY but not AMPA or mixture [255]. Nile tilapia (*O. niloticus*) exposed to GLY (0.6 mg l^{-1}) for 4 weeks indicated immunosuppression, an oxidative stress response, as well as liver and kidney dysfunction, as indicated by increased levels of glucose, cortisol, and enzyme activities (aspartate aminotransferase and alanine aminotransferase) in gills and other tissue samples [256]. The use of ginger in the feed showed a protective role by enhancing antioxidant and immunological responses in the exposed fish [256]. GLY (1, 5, and 10 mg l^{-1}), affected the energy metabolism and feeding behavior of *D. rerio* larvae leading to increased mortality [257]. The dynamics between zooplankton and fish larvae were severely affected by GLY, resulting in reduced survival and feeding rates. GLY was also found to bioaccumulate in zooplankton species, with levels up to 6.26% of the total weight of rotifers [257].

In juvenile *P. lineatus*, exposure to the GBH co-formulant POEA (0.15, 0.75, and 1.5 mg l^{-1}) resulted in increased plasma lactate levels and decreased hepatic catalase activity, red blood cell counts and hemoglobin content [258]. Additional effects included DNA damage, lipid peroxidation and hemolysis but with hematocrit levels not affected [258]. POEA (9.3 and 18.6 µg l^{-1}) was found to induce genotoxic effects in the European eel (*A. anguilla*) causing higher levels of DNA damage compared to GLY and a Roundup GBH. There was also a synergistic interaction between POEA and GLY in promoting non-specific DNA damage [221]. While no acute toxic effects of GLY and AMPA were observed on *D. rerio* embryos, significant lethal effects were detected after exposure to the GBH Atanor 48 and POEA. All tested compounds were found to be genotoxic based on Comet assays performed on zebrafish larval cells and rainbow trout gonad-2 (RTG-2) cells. Specifically, POEA induced DNA damage in RTG-2 cells in vivo, implying that it has direct genotoxic properties [259]. The different aquatic ecotoxicological studies demonstrated wide range of the possible side effects of GBHs and their components. The detected effects were indicated even at concentrations lower than environmentally relevant GLY levels ($\geq$0.01 mg l^{-1}). However, in several cases the effects are only seen at much higher concentrations. Based on the results, fish proved to be an excellent test organism for many endpoints such as DNA damage, oxidative stress, or the immune response (Table 8).

Effects on amphibians

Several studies have shown that GBHs at concentrations present and measured in the environment have adverse effects on amphibians (Table 9). The direct toxicity of GLY is often associated with higher doses or possibly the presence of GBH co-formulants. Lower concentrations of GLY have effects on tadpole development and behavior. The effects on amphibians are highly depend on the type and composition of GBHs and the sensitivity of different taxa and life stages. However, it is very difficult to determine applicable and valid environmental concentrations of GLY that occur in and affect amphibian habitats. Furthermore, little is known about the environmental concentrations of co-formulants in GBHs [260].

POEA, which has been banned as a co-formulant in GBHs in the EU since 2016 but is still widely used in the USA, is also toxic to the aquatic environment and amphibians due to its ability to disrupt membrane transport and act as a narcotic [261]. The toxic effects of GBHs on amphibians are often much higher than the toxicity of GLY alone. The toxicity of GLY and GBHs to amphibians and reptiles was also considered in EFSA's official scientific opinion on the risk assessment of commercial pesticide formulations [262]. Amphibians are a very specific group of animals that may be exposed to the effects of GLY and its commercial formulations in both aquatic and terrestrial habitats at different life stages, with amphibian reproduction generally dependent on and associated with water.

Chronic exposure to the GBH VisionMax (0.021–2.9 mg AI l^{-1}) decreased the number of wood frog (*Lithobates sylvaticus*) tadpoles that reached the metamorphic peak under laboratory conditions [263]. In addition, a concentration-dependent increase in thyroid hormone receptor β was observed in the brain of exposed tadpoles [263]. Not only the GBH Roundup Ultramax ($\geq$0.37 mg

Table 9 Effects of glyphosate, co-formulants, and/or its formulated herbicide products on amphibians reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
L. sylvaticus	VisionMax	0.021–2.9 mg AI[a] l^{-1}	48 d[b]	Survival, development, growth, sex ratio, gonadal morhology	Decreased survival, delayed metamorphic development (2.9 mg AI l^{-1}), affected weight and, tail length of tadpoles (≥ 0.021 mg AI l^{-1}), altered expression of thyroid- and stress-related genes, no effects on sex ratios or gonadal morphology	[263]
L. latrans	GLY[c], roundup ultramax	GLY: 3–300 mg l^{-1}, GBH[d]: 0.37–5.25 mg a.e.[e] GLY l^{-1}	96 h[f]	Growth, development, histopathological endpoints	No effects on growth and development, liver damage (GBH: ≥ 0.37 mg a.e. GLY l^{-1}, GLY: ≥ 15 mg l^{-1}), increased levels of melanomacrophagic cells and melanomacrophagic centers (GBH: ≥ 0.37 mg a.e. GLY l^{-1}, GLY: ≥ 3 mg l^{-1}), histopathological lesions	[82]
E. johnstonei	Roundup SL, surfactant Cosmoflux 411F	GBH mixture (55:44:1 – water:GBH:surfactant): in vivo toxicity: 7.6–112.3 µg a.e. GLY cm^{-2} (spraying), in vivo cito-and genotoxicity: 0.5–15 µg a.e. GLY cm^{-2}, in vitro cito-and genotoxicity: 4.6–37.0 mg a.e. GLY ml^{-1}	24–96 h	Mortality, cytotoxicity, genotoxicity	LC_{50}[g]males = 49 µg a.e. GLY cm^{-2} LC_{50}females = 54 µg a.e. GLY cm^{-2} LC_{50}neonates = 12 µg a.e. GLY cm^{-2} in vitro (≥ 95 µg a.e. GLY ml^{-1}) and in vivo (≥ 5.4 µg a.e. GLY cm^{-2}) cytotoxicity, dose-dependent effects on DNA damage	[264]
L. catesbeianus	GLY, GBHs (Roundup Original and Roundup Transorb)	1 mg a.e. GLY l^{-1}	96 h	Oxygen uptake, morphometric analysis	GLY and GBHs affected the oxygen uptake, caused changes in skin morphology, altered thickness of epidermis (GLY, Roundup Original)	[265]
X. laevis	GLY, GLY-IPA[h], isopropylamine, Roundup	0.002–1.69 g l^{-1}- (GLY equivalent)	24 h	Morphological analysis, melanosome aggregation, effects on microtubules, actin filaments and localization of melanosomes	Severe adverse effects on melanosome aggregation (116.4 mg l^{-1} GLY-IPA), pH-dependent effects of GLY, affected morphology, cytoskeletal integrity, and intracellular transport of melanosomes (0.085–0.845 g l^{-1})	[266]

Table 9 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
B. bufo	Roundup LB Plus,	0.5, 1.0, or 1.5 mg a.e. GLY l^{-1}	21–42 d	Egg and tadpole development	Temperature-dependent toxicity, exposure of eggs: increased tail, body, and total length; exposure of tadpoles: no effect on mortality, development, and morphology	[267]
B. bufo	Roundup PowerFlex	0–4 mg a.e. GLY l^{-1}	21–42 d	Egg and tadpole development	Temperature-dependent toxicity, exposure of eggs: increased tail and body length (15 °C); exposure of tadpoles: no effects on the tested parameters	[115]
B. spinosus	AMPA	0.4 µg l^{-1}	3 months	Hatching success, developmental abnormalities and morphology	No effects on hatching success, significant effects on fitness parameters (e.g., body and tail length)	[268]
B. spinosus	AMPA	0.07–3.57 µg l^{-1}	16 d	Hatching success, mortality and deformation rates	Higher embryonic mortality (0.07–0.39 µg l^{-1}), increased development duration, altered hatchlings morphology	[269]
L. pipiens, L. sylvaticus, B. americanus	Roundup Original MAX	1, 2, and 3 mg l^{-1} a.e. GLY	21 d	Survival and average individual mass, morphological analyses	LC_{50} = 2.44–3.26 mg l^{-1}, reduced mortality in presence of predators due to the induced antipredator morphology, interaction of GBH and predators on average mass	[270]
L. latinasus	GLY	170–17000 mg l^{-1}	48 h	Histological analysis, micronuclei and other erythrocytes nuclear abnormalities	Increased area of melanin, reduction in the area of hemosiderin, altered hepatic catabolism pigments, increased frequency of micronuclei (≥ 17,000 mg l^{-1}) and abnormalities in blood erythrocytes (17,000 mg l^{-1})	[271]

Table 9 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. cuvieri, P. gracilis	Roundup Original DI	Acute test:100–4500 µg a.e. GLY l^{-1}, chronic test: 65–1000 µg a.e. GLY l^{-1}	Acute test: 96 h, chronic test: 14 d	Mortality, length and mass, malformations	LC_{50} *P. cuvieri* = 1006 µg a.e. GLY l^{-1}, LC_{50}*P. gracilis* = 1131 µg a.e. GLY l^{-1}, shorter body length and lower body weight (≥ 500 µg a.e. GLY l^{-1}), morphological alterations	[272]
L. latrans	GLY, Roundup Ultra Max	GLY: 3–300 mg l^{-1}, GBH: 0.0007–9.62 mg a.e. GLY l^{-1}	24–96 h	Mortality, swimming activity, growth, development, morphological abnormalities	GLY: no effects on mortality, LC_{50} GBH = 3.26–9.61 mg a.e. GLY l^{-1}, affected growth and development, oral abnormalities and edema, altered swimming activity (GBH at the earlier developmental stage)	[273]
X. laevis	GBHs (Roundup, Kilo Max, and Environ Glyphosate)	Screening	96 h	Mortality	Earlier life stages of tadpole development showed higher sensitivity	[274]
X. laevis	GLY, Roundup Star	GLY: 282–500 mg l^{-1}, GBH: 31–50 mg AI l^{-1}	96 h	Mortality, development, enzyme activities	GLY has no effects on mortality, development, and enzyme activities, LC_{50} GBH = 32.1–35.1 mg AI l^{-1}, altered development (≥ 31 mg AI l^{-1}), reduced carboxylesterase (17.6 mg AI l^{-1})	[275]
X. laevis	Roundup LB Plus	Developmental assay (10–243 mg l^{-1}), neurological analysis and cardiotoxicity (121.5 and 243 mg l^{-1})	96 h	Mortality, mobility, development (cardiac, eye and brain)	No effects on mortality, developmental effects, altered mobility (≥ 97 mg l^{-1}), impaired cardiac (≥ 97 mg l^{-1}), eye and brain development (≥ 121.5 mg l^{-1})	[276]
X. laevis	GBHs (Roundup. Enviro Glyphosate, Kilo Max)	Roundup: 0.2–0.6 mg l^{-1}, Enviro Glyphosate: 0.9–28 mg l^{-1}, Kilo Max: 90–280 mg l^{-1}	96 h	Survival, development, sex ratio, reproductive malformation	No effects on mortality (GBHs), reduced body mass of the metamorphs (Kilo Max and Glyphosate Enviro), altered sex ratio (Kilo Max 280 mg l^{-1}), induced reproductive malformations	[277]

Table 9 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
E. cyanophlyctis	Roundup	Testing of survival: 1–8 mg a.e. GLY l^{-1}, genotoxicity study: 1–3 mg a.e. GLY l^{-1}	Testing of survival: 15 d, genotoxicity study: 24–96 h	Survival, genotoxicity	96-h LC_{50}GBH = 3.76 mg a.e. GLY l^{-1} and 3.39 mg a.e. GLY l^{-1} (in the absence and presence of predator), 10-d LC_{50}GBH = 2.12 mg a.e. GLY l^{-1} and 1.91 mg a.e. GLY l^{-1}, increased frequency of micronuclei (≥ 2.0 mg a.e. GLY l^{-1})	[278]
X. laevis	GLY, Roundup Power 2.0	GLY: 7.5–50 mg l^{-1} GBH: 1–25 mg a.e. GLY l^{-1}	96 h	Survival, LC_{50}, embryotoxicity, morphological analysis	Embryolethal effects of GLY (50 mg l^{-1}), 96-h EC_{50}^{i}GBH = 7.8 mg a.e. GLY l^{-1}, high teratogenic potential of GBH, with concentration-dependent abnormal phenotypes, cardiac malformations (≥ 30 mg l^{-1} GLY)	[279]
H. pardalis, P. cuvieri	GLY	84–150 mg l^{-1}	96 h	LC_{50}	LC_{50} = 106 and 115 mg l^{-1} for *P. cuvieri* and *H. pardalis*	[280]
D. minutus	Roundup Original	0.28–4 mg AI l^{-1}	96 h	Acute toxicity, DNA damage, micronucleus test	No mortality, no effects on body mass and length, increase in DNA damage, (≥ 0.28 mg AI l^{-1}), no significant effects on micronuclei in erythrocytes	[281]
R. dalmatina	Glyphogan Classic	2 and 6.5 mg a.e. GLY l^{-1}	21 d	Number of tadpoles, behavior	Reduced tadpole activity and increased hiding (6.5 mg a.e. GLY l^{-1}), altered vertical position (2 mg a.e. GLY l^{-1})	[282]
R. arenarum	GBHs (Roundup Ultra-Max, Infosato, and Glifoglex, C-K Yuyos)	1.85–240 mg a.e. GLY l^{-1}	48 h	Acute toxicity, enzyme activities	Differences in the acute toxicity of GBHs (similar effects of Glifoglex, C-K Yuyos), reduced activity of $AChE^{j}$, carboxylesterase, GST^{k}, and butyrylcholinesterase	[283]
B. bufo	Glyphogan Classic	2 and 4 mg a.e. GLY l^{-1}	Laboratory test: 9 d, outdoor mesocosm: 21 d	Bufadienolide content	Higher level of bufadienolides (4 mg a.e. GLY l^{-1}) during metamorphosis, increased bufadienolide content after three-weeks exposure in mesocosm (2–4 mg a.e. GLY l^{-1})	[284]

Table 9 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
L. sylvaticus	Roundup WeatherMax	(0.21 and 2.8 mg a.e. GLY l^{-1})	28–53 d	Survival, growth and development	GLY (2.8 mg a.e. GLY l^{-1}) and nutrient enrichment caused lower survival in situ, larger larvae, no significant effects on larval development	[285]
R. arenarum	Roundup Ultra-Max	20 mg l^{-1}	48 h	Enzyme activities, lipid peroxidation in erythrocytes,	No effects on butyrylcholinesteras, GST, and carboxylesterase activities or DNA damage, increased heterophil l-1ymphocyte (H l^{-1}) ratio in peripheral blood	[208]
A. maculatum	GLY-4 Plus	3 mg a.e. GLY l^{-1}	Toxicity test: 34 d, cellular immune response: 48 h	Survival, body size, percentage of metamorphs, and cellular immune response	Increased number of metamorphs (3 mg a.e. GLY l^{-1}) effects on survival, body size, and cellular immune response were strongly influenced by moderate or low UV-B light regimes	[286]
E. wilderae	Roundup	0.73–2.92 µg a.e. GLY l^{-1}	4–5 h	Behavioral parameters (e.g., exploratory movement frequency, distance, and refuge use)	Reduced burst distance, shorter and more frequent movements (2.92 µg a.e. GLY l^{-1}), effects were inconsistently affected by water temperature	[287]
D. minutus	GLY, POEA[l]	GLY: 65–520 µg l^{-1}, POEA: 1.25–10 µg l^{-1}	96 h	Genotoxicity, mutagenicity, hepatic histopathology	More genomic damage (1.25–10 µg l^{-1} POEA, ≥ 260 µg l^{-1} GLY), increased level of micronuclei (5 µg l^{-1} POEA, 520 µg l^{-1} GLY), increased DNA damage (5 µg l^{-1} POEA, ≥ 260 µg l^{-1}), histopathological effects	[288]
R. humboldti, E. pustulosus, H. crepitans, R. marina	Roundup Active, adjuvant Cosmo-Flux 411F	Laboratory test: 325–6000 µg a.e. GLY l^{-1}, microcosms: 3.69–29.52 kg a.e. GLY ha^{-1}	96 h	LC_{50}, body size parameters, swimming performance	Embryos were more tolerant, concentration-dependent effects on morphological parameters (e.g., total length, head width, corporal length), effects on embryonic development for *R. humboldti*, no effects on swimming performance	[289]

Table 9 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
B. fowleri, R. catesbeiana, H. chrysoscelis, R. clamitans, R. pipiens	GLY-IPA, Roundup, MON 0818	GLY-IPA: 0.42–41.48 mg a.e. GLY l^{-1}, GBH: 0.3–7.0 mg a.e. GLY l^{-1}, MON0818: 0.06–2.00 mg l^{-1}	96 h	Mortality	No significant mortality of GLY, LC_{50}GBH = 1.80–4.22 mg a.e. GLY l^{-1}, LC_{50}POEA = 0.68–1.32 mg l^{-1}	[290]
R. dalmatina, B. bufo	GLY, POEA	GLY: 1, 2 or 4 mg a.e. GLY l^{-1}, POEA: 0.44, 0.88 or 1.74 ml l^{-1}	96 h	Survival, body mass	Reduced survival and affected bod mass (≥ 0.88 ml l^{-1} POEA), mortality and body mass were not affected by GLY alone, in the presence of the POEA, higher mortality	[291]

[a] Active ingredient, [b]day, [c]glyphosate, [d]GLY-based herbicides, [e]acid equivalent, [f]hour, [g]50% lethal concentration, [h]GLY-isopropylammonium salt, [i]50% effective concentration, [j]acetylcholinesterase, [k]glutathione-*S*-transferase, [l]a mixture of polyethoxylated tallow amines

a.e. GLY l^{-1}), but also GLY (≥ 15 mg l^{-1}) caused liver damage in neotropical frog (*Leptodactylus latrans*) tadpoles at concentrations frequently found in the environment [82]. Cytotoxic effects of a GBH mixture (Roundup SL and surfactant Cosmoflux 411F) have been demonstrated in various in vitro (at concentrations from 95 µg a.e. GLY ml^{-1}) and in in vivo (at application rates above 5.4 µg a.e. GLY cm^{-2}) tests on Antilles coqui *Eleutherodactylus johnstonei* erythrocytes with a dose-dependent induction of DNA breaks [264]. Exposure to a sublethal concentration of GLY (1 mg l^{-1}) and GBHs (Roundup Original and Roundup Transorb at 1 mg a.e. GLY l^{-1}) caused skin changes and altered respiratory function in bullfrog (*Lithobates catesbeianus*) tadpoles [265]. Differences can be observed in the effects of the GBH formulations compared to the effects of GLY alone, and even differences can be observed in the toxicity of the GBHs [265]. In the African clawed frog (*Xenopus laevis*), severe adverse effects on melanosome aggregation were observed at low concentrations (116.4 mg l^{-1}) of GLY-IPA compared to treatment with a Roundup GBH [266]. The effects of GLY were pH dependent, in contrast to the effects of the formulation. Roundup affected the morphology, cytoskeletal integrity, and intracellular transport of melanosomes in the exposed animals [266]. A study conducted on the common toad (*Bufo bufo*) exposed to a GBH (Roundup LB Plus at 0.5, 1.0, or 1.5 mg a.e. GLY l^{-1}) at two different temperatures (15 °C or 20 °C) and life stages (eggs or tadpoles) found that eggs were more sensitive compared to tadpoles [267]. More pronounced toxicity of GBH, particularly on egg development, was observed at the lower temperature which may be due to interactive effects of the factors tested. Exposure of eggs to GBH resulted in an average 31% increase in tail, body, and total length compared to controls. Effects on mortality, development, or morphology were not observed in the exposed tadpoles [267]. The effects of the GBH Roundup PowerFlex (1.5–4 mg AI l^{-1}) were studied on the larval development of *B. bufo* exposed in different life stages (eggs or tadpoles) were studied at two different temperatures (15 °C and 20 °C). Exposure of eggs resulted in significantly increased tail and body length, but only at the lower test temperature [115]. No effects were observed on mortality, body weight, and condition of the exposed tadpoles. Nevertheless, significant interactions between GBH and temperature on tadpole developement, larval tail length, body length and width were observed [115]. Additionally, strong adverse effects of AMPA at early developmental stages (0.4 µg l^{-1}) were detected toads [268]. Moreover, altered hatchling morphology, increased embryonic mortality and longer development duration in *Bufo spinosus* were observed following exposure to AMPA (0.07–0.39 µg l^{-1}) [269].

Roundup Original MAX (with POEA as a co-formulant) resulted in morphological changes in tadpoles of Northern leopard frog (*Lithobates pipiens*), wood frog (*L. sylvaticus*), and American toad (*Bufo americanus*) [270]. Frog tadpoles exhibited relatively deeper tails, and the presence of predators reduced the mortality observed in the presence of Roundup Original MAX because the herbicide induced antipredator morphology [270].

Exposure to pure GLY (100–0000 µg g^{-1}) caused morphological changes in the liver of the oven frog (*Leptodactylus latinasus*) [271]. GLY increased the melanin area in liver melanomacrophages, altered the presence of hepatic catabolism pigments into melanomacrophages, and also caused abnormalities of blood erythrocyte nuclei [271]. In addition to lethal effects, shorter body length and lower body weight were observed in tadpoles of native South American frogs (*Physalaemus cuvieri* and *P. gracilis*) exposed to the GBH Roundup Original DI (≥ 500 µg a.e. GLY l^{-1}) [272]. Growth and development of *L. latrans* were affected by GLY (3–300 mg l^{-1}) and its formulation a Roundup GBH (0.0007–9.62 mg a.e. GLY l^{-1}). Oral abnormalities and edema were observed after exposure to both substances, while swimming activity was altered only by Roundup treatment at the earlier developmental stage of tadpoles [273]. At earlier life stages of tadpole development, *X. laevis* showed higher sensitivity to the toxic effects of GBHs, such as Roundup formulations, with the pre-metamorphic stage being the most sensitive [274]. GLY had no developmental or lethal effects on *X. laevis* embryos and tadpoles up to 500 mg l^{-1}, whereas the GBH Roundup Star adversely affected embryos and tadpoles even at much lower concentrations (≥ 31 mg AI l^{-1}) [275]. Exposure to sublethal concentrations of the GBH Roundup LB Plus resulted in decreased body length and mobility of *X. laevis* larvae (≥ 97 mg l^{-1}) [276]. This GBH also impacted heart development, including decreased heart rate and atrium size (≥ 97 mg l^{-1}). Additionally, smaller eyes, cranial cartilages, brains, and shorter cranial nerves were observed after treatment (≥ 121.5 mg l^{-1}) [276]. A significant decrease in body mass of *X. laevis* metamorphs was observed after exposure to GBHs (Kilo Max and Enviro). Kilo Max (280 mg l^{-1}) altered the sex ratio of exposed frogs (68:32–F:M) compared to controls (50:50). Reproductive malformations, such as translucence, mixed sex, and aplasia, were also observed [277].

Lethal and genotoxic effects of Roundup were observed in the South Asian frog species *Euflictis cyanophlyctis*, with observed effects increasing in the presence of predation stress [278]. Sublethal and teratogenic effects of the GBH Roundup Power 2.0 were observed in embryos of *X. laevis*, while a dose-dependent abnormal phenotype, including microphthalmia

craniofacial alterations, arrow eyes, and forebrain regionalization defects, was induced after treatment, which can be explained by GLY penetration facilitated by the surfactant co-formulants (1–25 mg a.e. GLY l^{-1}) [279]. Additionally, cardiac malformations were indicated after GLY exposure (≥ 30 mg l^{-1}) [279]. Minor differences in the sensitivity of the tropical frog species studied (*Hypsiboas pardalis* and *Physalaemus cuvieri*) were observed in GLY toxicity tests, as indicated by the 96-h LC_{50} values (106 and 115 mg l^{-1} for *P. cuvieri* and *H. pardalis*, respectively) [280]. A lower concentration of the GBH Roundup Original (≥ 0.28 mg AI l^{-1}) significantly increased DNA damage in *D. minutus* tadpoles [281]. Exposure to the GBH Glyphogan Classic (2 and 6.5 mg a.e. GLY l^{-1}) caused behavioral changes in tadpoles of the agile frog *Rana dalmatina* [282]. At higher concentrations, reduced tadpole activity was observed with more tadpoles hiding. At the lower concentration tested, the vertical position of the tadpoles was closer to the water surface than in controls. In addition, some of the observed behavioral changes resembled the movements induced by the presence of predators, such as dragonfly larvae [282]. The effects of various GBHs (including Roundup Ultra-Max, Infosato, Glifoglex, and C-K Yuyos) on enzymatic parameters (such as reduced activity of AChE, carboxylesterase, GST, and butyrylcholinesterase), were demonstrated in tadpoles of *Rhinella arenarum* [283]. Tadpoles of *B. bufo* exposed to Glyphogan Classic GBH (4 mg a.e. GLY l^{-1}) throughout larval development showed a higher amount of bufadienolides during metamorphosis compared to the control group [284]. Wood frog (*L. sylvaticus*) larvae exposed to the GBH Roundup WeatherMax (0.21 and 2.8 mg a.e. GLY l^{-1}) had larger larvae, but no significant effects on larval development were observed [285]. Exposure to a Roundup Ultra-Max GBH (20 mg l^{-1}) did not resulted in increased induction of DNA damage, oxidative stress or neurotoxicity. In addition, enzyme activities (e.g., butyrylcholinesteras, GST, and carboxylesterase activities) were not altered either. However, an increased heterophil ʟ-1ymphocyte (H/L) ratio in peripheral blood was detected indicating immunological depression in *R. arenarum* [208].

Based on the results within an artificial pond mesocosm, the effects of the GBH GLY-4 Plus on survival, body size, and cellular immune response of spotted salamanders (*Ambystoma maculatum*) were strongly influenced by the applied UV-B light regimes (moderate or low) [286]. In larval salamanders (*Eurycea wilderae*) exposed to GBHs such as Roundup, shorter and more frequent movements were observed at higher GLY concentrations, while GLY-induced effects were inconsistently affected by water temperature [287].

Genotoxic, mutagenic, and histopathological hepatic effects of POEA and GLY were observed in lesser treefrog (*D. minutus*) tadpoles [288]. More genomic damage (174%) was observed in POEA-exposed tadpoles at all concentrations (1.25–10 µg l^{-1}) compared to controls. Additionally, up to a sevenfold increase in micronuclei was recorded on average at 5 µg l^{-1} POEA. All individuals exposed to 10 µg l^{-1} POEA died. GLY exposure increased DNA damage by 165% at higher concentrations (260 and 520 µg l^{-1}) and also gave rise to more micronuclei (up to sixfold) at 520 µg l^{-1} [288]. The mixture of the GBH Roundup Active and the surfactant Cosmo-Flux 411F caused concentration-dependent sublethal effects on the body size of tadpoles (e.g., *Rhinella humboldti*, *Engystomops pustulosus*, *Hypsiboas crepitans*) [289]. However, significant effects on embryonic development were observed only on *R. humboldti*. It was noted that embryos appeared to be significantly more tolerant compared to tadpoles, which may be explained by the exclusion of the chemical compounds of the embryonic membranes and the absence of surfactant-sensitive organs, such as the gills [289]. Alterations of swimming performance were not observed in the investigated microcosms [289]. Exposure to surfactant MON 0818 (POEA) resulted in 96-h LC_{50} values ranging from 0.68 to 1.32 mg l^{-1} in the North American anuran species (e.g., *Rana pipiens*, *Rana clamitans*, and *Hyla chrysoscelis*), indicating differences in the sensitivity of anuran species to this GBH co-formulant [290]. Most of the presented studies highlight that co-formulants are the main cause of high-level toxicity of pesticide formulations to amphibians. Similarly, in acute toxicity testing on *R. dalmatina* and *B. bufo* tadpoles, the mortality and body mass were not affected by GLY [283]. However, in the presence of the POEA, higher mortality was observed in both species with high toxicity of POEA alone was also demonstrated [291]. The results of the ecotoxicological studies on amphibians indicated several alterations in the physiological, morphological and metabolic parameters. Several effects were detected even at environmentally relevant GLY concentrations, demonstrating the particular vulnerability of amphibians (Table 9).

Combined effects between glyphosate and other environmental pollutants

The various chemical compounds (e.g., pesticide AIs, formulation agents, pharmaceutical residues) present in the different environmental matrices in all likelihood will come into contact with each other. GLY and its metabolites (e.g., AMPA) will coexist in the aquatic environments with the other aquatic pollutants. Therefore, identifying and evaluating the potential combined effects of these various pollutants is essential to conducting a

comprehensive ERA for commercial pesticide formulations, including GBHs. The presented combined effects between GLY and other environmental pollutants are summarized in Tables 10 and 11.

Combined effects with other AIs, co-formulants, and other aquatic pollutants

The concern about ecotoxicological consequences and thus adverse effects of pesticide residues stem from possible additive or synergistic effects of combinations of various compounds of agricultural (and other) origin. Multi-and transgenerational synergistic effects of GLY and chlorpyrifos were observed in the estuarine rotifer *P. similis* exposed to the mixture of tested AIs at environmentally relevant concentrations [181]. Reduced growth was observed in generations F0 to F6, but the transgenerational effects were eliminated in F5, indicating a slight recovery and population resilience to pollution [181]. Simultaneous exposure of crayfish (*Pontastacus leptodactylus*) to the insecticide chlorpyrifos and GLY for 21 days resulted in synergic effects with an increase in glutamic-oxaloacetic-transaminase activity and total antioxidant content, while γ-glutamyltransferase (GGT) activity decreased whilst exposure to GLY alone increased GGT activity in *P. leptodactylus* [292]. The potential adverse effects of GLY (3.5 mg l^{-1}) and chlorpyrifos (25 µg l^{-1}) were assessed individually and in combination on common carp (*C. carpio*) over 21 days [293]. In addition to induced accumulation of malondialdehyde in the brain, decreased enzyme activities (e.g., AChE, catalase, GST) were observed after exposure to the test substances individually. In combination, the impact on most parameters measured were enhanced over that observed for the individual compounds suggesting that exposure to the investigated AIs both individually and in combination, may lead to oxidative stress and lipid peroxidation in common carp [293]. In addition, changes in the transcriptome were also detected in fish brains after treatment with GLY and chlorpyrifos in fish brains but again enhanced with the mixture of the two [293]. A synergistic effect of a mixture of the GBH Credit (50.0–100.0 mg l^{-1}) and the dicamba-based commercial herbicide formulation Banvel (96.0–720.0 mg l^{-1}) was demonstrated in the induction of primary DNA breaks in circulating blood cells of late-stage *R. arenarum* larvae [294]. Exposure to a higher concentration of the combined herbicides caused a significant increase in genetic damage index (GDI) [294]. Similarly, an increased GDI was observed with a combination of the Credit GBH and imazethapyr-based (Pivot) herbicides on *R. arenarum* tadpoles [295]. After co-exposure to the herbicides, synergistic effects were demonstrated in DNA damage induction based on measurements in blood cells compared to treatment with the single herbicide [295]. GLY and 2,4-D are the most commonly used herbicides worldwide with well over 700,000 and 150,000 tonnes applied per year, respectively [296], and are used singly and in combination for weed control in various crops such as cotton, soybean, and corn [297, 298]. Therefore, these two herbicide AIs are frequently detected in surface waters, especially near agricultural fields [35, 299–303]. The combination of GLY and 2,4-D had no effect on the survival of exposed *Boana faber* and *L. latrans* tadpoles although swimming activity and growth were significantly affected [303]. Additionally, various types of damage and abnormalities were observed in the intestine, mouth, and erythrocytes of tadpoles [303].

While the mechanism of how co-formulants enhance the uptake of pesticide AI is well known [17, 304], predicting negative impacts on the non-target organisms is not straightforward. Furthermore, conducting ecotoxicological testing on various co-formulants is difficult, as these components are usually not identified on the labels of commercial pesticide formulations with their exact composition often considered as confidential business information. Many studies have shown that co-formulants of GBH can affect toxicity, including phytotoxicity, cytotoxicity developmental neurotoxicity, genotoxicity, and endocrine-disrupting effects of GLY on various non-target organisms such as fish and amphibians [31, 32, 74, 175, 247, 277, 305, 306].

Other environmental pollutants, such as heavy metals, nanomaterials, and microplastics may be present in aquatic environments, and these chemical compounds may also interact with GLY residues and its metabolites. A concentration-dependent effect of a combination of copper and GLY on the growth and physiological response of *Salvinia natans* has been reported [307]. Antagonistic effects were observed in plants exposed to low concentrations of copper and GLY, while synergistic effects were observed at higher concentrations. Furthermore, higher levels of hydrogen peroxide malondialdehyde were detected after individual and combined exposure, indicating the occurrence of oxidative stress [307]. After exposure to a GBH (Faena, 1.04–1.57 mg l^{-1} GLY) and copper (2.45–4.31 µg l^{-1}), a delayed age at first reproduction, an increased number of aborted eggs, reduced fecundity and a lower number of clutches per female were observed in the parental and F1 generations of *Daphnia exilis* [165]. In addition, reduced carbohydrate and lipid contents were detected in both generations [165]. The observed combined effects of GLY and copper were stronger in the F1 generation [165]. Due to the presence of arsenic in natural phosphate ores, their use in the production of agrochemicals and particularly phosphate fertilizers, may pose an additional risk to the

Table 10 Combined effects of glyphosate and/or its formulated herbicide products with other aquatic pollutants reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. similis	Chlorpyrifos and GLY[a]	acute test: 1–60 mg l^{-1} GLY and 50–1000 µg l^{-1} chlorpyrifos, chronic test: 1–1000 µg l^{-1} GLY and 0.1–10 µg l^{-1} chlorpyrifos	48 h[b]	Acute toxicity, multigenerational effects	Reduced growth of F0 to F6 generations, transgenerational effects	[181]
P. leptodactylus	Chlorpyrifos-based and GLY-based formulations	GLY: 0–0.8 mg l^{-1}, chlorpyrifos: 0–5 µg l^{-1}	21 d[c]	Combined effects on biochemical and immunological parameters in hemolymph, oxidative biomarkers in hepatopancreas	Decreased enzyme activities (e.g., AChE[d] and alkaline phosphatase and total protein levels, increased enzyme activities (e.g., glutamic-pyruvic-transaminase), glucose content and malondialdehyde levels	[292]
C. carpio	Chlorpyrifos and GLY	CPF[e]: 25 µg l^{-1}, GLY: 3.5 mg l^{-1}, alone and in combination	21 d	Enzyme activites (AChE, Na^+/K^+-ATPase, monoamine oxidase), stress-related indicators (e.g., SOD[f], CAT[g], GST[h])	Induced accumulation of malondialdehyde in the brain, decreased enzyme activities (e.g., AChE, catalase, GST) were observed after individual exposures, increased combined toxicity	[293]
R. arenarum	Dicamba-based (Banvel) and GLY-based (Credit) formulations	Banvel 96.0–720.0 mg l^{-1}, GBH[i]: 50.0–100.0 mg l^{-1}	96 h[i]	Mortality, genotoxic effects (DNA damage)	LC_{50}[j] = 358.44 mg l^{-1} and 78.18 mg l^{-1} for dicamba and GLY, induced DNA damage, exposure, increased genetic damage index, higher combined toxicity	[294]
R. arenarum	Imazethapyr-based (Pivot) and GLY-based (Credit) formulations	Credit: 3.91 and 7.82 mg l^{-1} GLY, Pivot: 0.05 and 0.10 mg l^{-1} imazethapyr	96 h	Mortality, DNA damage	LC_{50} = 78.18 mg l^{-1} GLY and 0.99 mg l^{-1} imazethapyr, increased GDI[k] and DNA damage after individual and combined exposures, increased combined toxicity	[295]
B. faber and L. latrans	GLY-based (Roundup Original DI) and 2,4-D-based (Nortox) formulations	GLY: 65, 144, 280, 500, 1000 µg l^{-1}; 2,4-D: 4, 17, 30, 50, 74.5 µg l^{-1} in combinations	7 d	Survival, body size, swimming activity, morphological effects, micronuclei and erythrocytes nuclear abnormalities	No effects on survival, altered length and body mass of *L. latrans,* affected swimming activity, erythrocyte nuclear abnormalities and damages in the intestine and mouth of tadpoles	[303]

Table 10 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
S. natans	Cu^{2+} and GLY	Cu^{2+}: 0, 0.2, 1, 5, 8, 10 mg l^{-1}, GLY: 0, 1, 5, 25, 50, 75 mg l^{-1}	7 d	Growth, morphological parameters, photosynthetic pigments, oxidative stress indicators, antioxidative enzyme activities (e.g., SOD, CAT)	Concentration-dependent combined effects on growth and physiological responses, antagonistic effects at low concentrations of copper and GLY, while synergistic at higher concentrations, increased level of malondialdehyde (Cu^{2+}: 0.2–5 mg l^{-1}, GLY: 1–25 mg l^{-1})	[307]
D. exilis	Cu^{2+} and Faena	1.04–1.57 mg l^{-1} GLY and 2.45–4.31 µg l^{-1} Cu^{2+} in combination	21 d	Survival, fecundity, macromolecule biomarkers, size of neonates	Decreased fecundity and number of clutches per female, delayed time at first reproduction, increased number of aborted eggs, reduced carbohydrate and lipid content, no effects on total length, decreased body length and width	[165]
D. magna	Binary mixture of GLY and silver nanoparticles)	GLY: 0–120 mg l^{-1}, (acute testing), 2.92–14.86 mg l^{-1} (chronic testing); silver nanoparticles: 0–0.3 µg l^{-1} (acute testing), 0.017–0.09 µg l^{-1} (chronic testing)	48 h acute tests, 21 d chronic test	EC_{50}^{I}, reproductive parameters (e.g., release of the firs offspring, number of newborns	48-h EC_{50}GLY = 89.2 mg l^{-1}, 48-h EC_{50} silver nanoparticles = 0.18 µg l^{-1}, higher combined toxicity on reproductive parameters (e.g., delayed release of the first offspring), no clear interaction in the combined multigenerational effects	[173]
P. reticulata	Maghemite nanoparticles (NPs^{m}) and a GBH (Roundup Original)	NPs: 0.3 mg l^{-1}, GBH: 65 and 130 µg GLY l^{-1} in two combination	14 and 21 days	Genotoxicity and mutagenicity (DNA damage, nuclear alterations)	Clastogenic (DNA damage) and aneugenic (cell nuclear alterations) time-dependent effects, synergistic effects	[311]
O. niloticus	GBH (Roundup) and propolis nanoparticles	GBH (0.6 mg AI^{n} l^{-1}), propolis nanoparticles: 10 g l^{-1}	4 weeks	Growth, oxidative stress biomarkers, serum biochemical and immunological parameters	No effects on growth performance, altered immunological parameters, liver and kidney indicators, induction of oxidative stress, inhibited AChE activity, decreased white and red blood cell counts	[312]

Table 10 (continued)

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
P. reticulata	Iron oxide NPs, GLY, GBH (Roundup Original)	NPs (0.3 mg l^{-1}) in combination with GLY (0.65 mg l^{-1}) or GBH (0.65 and 1.30 mg l^{-1})	21 d	Histopathological and histochemical parameters	Circulatory disturbances, activation of the immune system, inflammatory responses, decreased glycogen reserve, concentration-dependent ultrastructural changes	[313]
M. aeruginosa	GLY and polystyrene cationic amino-modified nanoparticles in combination	GLY: 0.5, 1, 3, 5, 7 mg l^{-1}, polystyrene nanoparticles: 3, 5, 10 and 20 mg l^{-1} in combination	48–96 h	Growth, adsorption capacity for GLY, microcystin level	Antagonistic combined toxicity of GLY and polystyrene nanoparticles, high adsorption capacity of nanoparticles for GLY, no effects on total microcystin level	[314]
D. magna	Polystyrene nanoplastic and GLY	polystyrene nanoplastic: 15.6, 31.2, 62.5, 125, 250, and 500 mg l^{-1}; GLY: 6.2, 12.5, 25, 50, 100, and 200 mg l^{-1} in combination	48 h (acute test), 21 d (chronic test)	Acute toxicity, ROS[o] production, swimming behavior, reproduction	Synergistic effects on acute toxicity, increased immobility and ROS production, reduced swimming activity, altered multigenerational responses and reproductive parameters	[315]
D. magna	GLY acid, GLY-IPA[p], Roundup Gran in combination with two types of microplastics (polyethylene microbeads and polyethylene terephthalate/polyamide fibers)	GLY: 2.5 mg l^{-1}, microplastics: 2.2*10^6 ml^{-1} (microbeads), 10–30 ml^{-1} (fiber)	7 d	Mortality	Increased mortality of GLY in the presence of microplastics	[316]
C. carpio	Polyethylene microplastics and GLY	GLY: 0, 5, 15 mg l^{-1}, microplastics: 0, 1.5, 4.5 mg l^{-1} in combination	60 d	Growth, AChE activity, behavior parameters, microbiome	Inhibited growth and free-swimming behavior (15 mg l^{-1} GLY and 4.5 mg l^{-1} microplastics, no effects on blood–brain barrier permeability and AChE activity, damaged physical and chemical intestinal barriers, altered gut microbiota	[252]

[a] Glyphosate, [b]hour, [c]day, [d]acetylcholinesterase, [e]chlorpyrifos, [f]superoxide dismutase, [g]catalase, [h]glutathione-*S*-transferase, [i]GLY-based herbicides, [j]50% lethal concentration, [k]genetic damage index, [l]50% effective concentration, [m]nanoparticles, [n]active ingredient, [o]reactive oxygen species, [p]GLY-isopropylammonium salt

Table 11 Combined effects of glyphosate and/or its formulated herbicide products with pathogens or parasites reported in the scientific literature since 2010

Test organism	Type of tested compound	Tested concentrations	Duration	Tested endpoints	Observed effects	References
O. mykiss	GLY[a], GBHs[b] (Roundup Innovert, Viaglif Jardin) in combination of infectious hematopoietic necrosis virus	1 µg l^{-1} AI[c] equivalent	8 months	Behavior, clinical signs of rhabdoviruses infection, and mortality, oxidative stress parameters, energy metabolism markers and immune markers	GLY and GBHs affected the susceptibility of fish to viral infection, substance-specific effect on cumulative mortality, no significant effects on most of the tested endpoints	[317]
O. mykiss	GLY, GBHs (Roundup Innovert, Viaglif Jardin) in combination of infectious hematopoietic necrosis virus	500 µg l^{-1} AI equivalent	96 h[d]	Mortality, metabolic and hemato-immunologic parameters, ability to survive a viral challenge	Significant differences in red and white blood cell counts, altered enzymatic activities in the infected fish, multistress conditions	[318]
G. anomalus	Glyphosate 360 formulation combined with *Telogaster opisthorchis* infection	0.36, 3.6, 36 mg AI l^{-1}	26 d	Survival, development, spinal malformations	Simultaneous exposure to GLY and parasitic infection significantly decreased fish survival (0.36 mg AI l^{-1}), spinal malformations	[319]
L. rohita	Roundup combined with *Aeromonas hydrophila* infection	0.63, 1.03, and 2.06, 13.6 mg AI l^{-1}	96 h	Susceptibility test	Reduced survival, increased vulnerability to infections	[222]

[a] Glyphosate, [b]GLY-based herbicides, [c]active ingredient, [d]hour

environment and food safety [18, 308]. A worrying finding in this context is the detection of heavy metal (e.g., arsenic, chromium, nickel, lead) impurities and petroleum residues in 11 different GBHs (e.g., Glyphogan, Medallon Premium, Roundup Classic) [18]. The presence of impurities (e.g., heavy metal, residues of polycyclic aromatic hydrocarbons), may originate from the production phase of the commercial formulations and potentially can contribute to the toxicity of GBHs (e.g., possible endocrine disrupting effects, carcinogenicity, neurotoxicity) [18, 309, 310]. Therefore, regulators should require manufacturers to identify and quantify toxic impurities in commercial pesticide formulations.

In chronic tests, toxic multigenerational effects of a mixture of GLY and silver nanoparticles were observed in *D. magna* [173]. A significant delay in the release of the first offspring and altered reproductive parameters (reduced number of newborns) were also demonstrated in the unexposed and offspring exposed to the individual compounds. Simultaneous exposure to GLY and silver nanoparticles resulted in a higher degree of toxicity compared to that observed with the individual test substances. In acute toxicity testing, antagonistic and additive interactions were observed, possibly due to GLY forming complexes with the nanoparticles [173]. Simultaneous exposure of citrate-functionalized iron oxide nanoparticles and the GBH Roundup Original resulted in clastogenic (DNA damage) and aneugenic (cell nuclear alterations) time-dependent effects in guppies (*P. reticulata*) [311]. Synergistic effects were observed compared to controls and guppies exposed to nanoparticles alone [311]. In Nile tilapia (*O. niloticus*) exposed to a Roundup GBH (0.6 mg AI l^{-1}), the toxic effects of GLY, such as induction of oxidative stress and immunosuppression were alleviated in the presence of propolis nanoparticles fed to exposed animals compared to the GLY-alone exposed group. This was evident through reduced gill and liver glutathione concentrations and decreased white and red blood cell counts [312]. Circulatory damages, inflammatory responses, and the activation of the immune system were observed in *P. reticulata* exposed to the mixture of a GBH (Roundup Original) and iron oxide nanoparticles [313]. Additionally, concentration-dependent ultrastructural alterations were observed [313].

In the environment, plastic waste can undergo degradation processes that lead to the formation of micro- and nano-plastics. These micro- and nano-plastics can directly and indirectly affect aquatic organisms, and can adsorb other chemical compounds, leading to combined contamination. The antagonistic combined toxicity of GLY and polystyrene nanoparticles modified with cationic amino acids, was observed in the inhibition of the growth of blue-green algae (*M. aeruginosa*) [314]. This was attributed to the high adsorption capacity of nanoparticles for GLY, resulting in a lower inhibitory effect of this herbicide AI. The presence of GLY increased the stability of the dispersion system, allowing for higher adsorption of nanoparticles on the surface of algal cells, which may lead to biomagnification of nanoparticles in food webs [314]. Synergistic effects were demonstrated in *D. magna* exposed to a combination of GLY and polystyrene nano-plastic [315]. Simultaneous exposure of the tested compounds resulted in increased immobility and production of ROS, while swimming activity decreased. Multigenerational responses were also observed after exposure of the parental (F_0) generation of daphnids to the mixture of GLY and nano-plastic, with altered reproductive parameters in the F_1 and F_2 generations as indicated in recovery tests [315]. The tested GLY forms (GLY acid, GLY-IPA, and GBH Roundup Gran) also increased the mortality of *D. magna* in the presence of microplastics such as polyethylene microbeads and polyethylene terephthalate/polyamide fibers, while the interaction between the treatment and time was not significant [316]. After 60 days of exposure to a combination of GLY (15 mg l^{-1}) and polyethylene microplastics (4.5 mg l^{-1}), free-swimming behavior of *C. carpio* was found to be inhibited [252]. Microplastics alone and in combination with GLY disturbed physical and chemical intestinal barriers in exposed fish. Altered abundance and diversity of the gut microbiota and changes in amino acid and lipid metabolism were also observed with simultaneous exposure to the test compounds [252].

Combined effects with pathogens and parasites

Exposure to a low concentration (1 µg l^{-1}) of GLY and GBHs (Roundup Innovert and Viaglif Jardin) had an impact on the susceptibility of rainbow trout *O. mykiss* fish to viral infection, specifically to hematopoietic necrosis virus [317]. Roundup Innovert significantly reduced cumulative mortality, while exposure to Viaglif Jardin resulted in increased mortality of *O. mykiss*, whereas pure GLY had little effect on the endpoints studied [317]. Furthermore, exposure to a higher concentration (500 µg l^{-1}) of GLY or its GBH formulations caused significant differences in red and white blood cell counts and altered enzymatic activities in *O. mykiss* infected with infectious hematopoietic necrosis virus after a 96-h exposure and 96-h post-viral infection [318].

Individual exposure to a GBH (0.36 mg AI l^{-1}) and the trematode parasite *Telogaster opisthorchis*, did not affect the survival of juvenile roundhead galaxias (*Galaxias anomalus*) fish [319]. However, simultaneous exposure to GLY and parasitic *T. opisthorchis* infection significantly decreased fish survival. Juvenile fish exhibited spinal malformations after exposure to the infection alone and

in combination with GLY, and synergistic effects were observed between GLY and the presence of parasites. GLY at a moderate concentration (3.6 mg AI l^{-1}) resulted in significantly higher production of *T. opisthorchis* cercariae in their snail intermediate host, the New Zealand mud snail (*Potamopyrgus antipodarum*), compared to the control group [319]. In the fish *L. rohita*, a significantly increased susceptibility to the pathogen *Aeromonas hydrophila* was observed in the presence of a GBH at sub-lethal concentrations (Roundup, 0.63–13.6 mg AI l^{-1}) [222]. Therefore, reduced survivability and increased susceptibility to the infection was observed in GBH exposed fish [222].

The detected interactions between GLY/GBH and other environmental pollutants are immensely complex effects. The presented combined effects between GLY and pathogens or parasites are summarized in Table 11. The combined toxicity of various chemical compounds is understudied, whilst during the ERA regulatory agencies generally rely on results obtained solely from standard laboratory studies using test organisms exposed to a range of concentrations of single compounds. However, under natural conditions, organisms come into contact with a very wide range of environmental pollutants. From the studies presented here, it appears that numerous aquatic pollutants can alter the effects of GLY and GBHs.

Comparison with the 2023 EFSA conclusion on aquatic toxicity of GLY/GBH

The most recent conclusion on the peer review of the risk assessment of GLY was published by EFSA on 26 July 2023 [320]. The document provides an evaluation of the risk profile of GLY based on undisclosed studies submitted by the manufacturers and the publicly available peer-reviewed scientific literature. According to the conclusions, the overall data provided in the risk assessment of GLY were considered sufficient for the assessment of environmental exposure, but concerns were raised about the potential exposure of groundwater via infiltration or contaminated surface water bodies due to the large proportion of land treated with GLY. This was recognized as a data gap. Furthermore, the surface water monitoring for GLY and AMPA residues carried out by the applicants, showed weaknesses in methodology and the use of minimum quality criteria and was, therefore, considered to have limited suitability for regulatory purposes. These issues are critical as they may impact the ecological health and the safety of water sources. Overall, the EFSA conclusion highlights general data gaps and potential risks and refers to the lack of harmonized methods and sufficient data on the adverse effects on aquatic macrophytes, broader ecological impacts, or the aquatic stage of amphibians. In addition to these uncertainties, the assessment does not conclude on certain areas such as adverse effects on biofilms or changes in microbial communities.

Therefore, our review aquatic ecotoxicology on different groups of aquatic organisms (from microbial communities, cellular and high-ordered macrophytes to aquatic invertebrates and vertebrates) is an essential complement to adequately assess the impact of increased use of GLY/GBHs [320]. Based on EFSAs conclusion and the results of the reported ecotoxicological studies, it is essential to develop state of the art guidelines to adequately address all environmental hazards from the use of GLY/GBHs, including the most sensitive species. According to the EU Pesticide Law (Regulation (EC) 1107/2009), the same level of safety should be ensured for a pesticide product as for the AI. Pesticide exposure under real environmental conditions occurs in the form of commercial pesticide formulations, but is only taken into account in the EU in a second stage at the Member State level. To comply with EU legislation and protect human health and the environment, studies on AIs and formulations should be considered during the risk assessment for the authorization of AIs.

In addition, stricter regulation of co-formulants per se is needed, as a co-formulant can affect the toxicity of the formulation and the fate of the AI in the environment. However, Annex III of Regulation (EC) 1107/2009, which is supposed to contain the list of banned co-formulants in commercial pesticide formulations, still does not contain an entry [36]. This is difficult to understand from a scientific point of view, as there is ample evidence for the acute and chronic toxicity of this class of substances. Moreover, a standardized approach should be developed to assess the combined toxicity of different co-occurring chemical compounds. The ecotoxicological assessment of the individual co-formulants and the combined effects of the components contained in formulated products should be an essential part of a comprehensive ERA for commercial pesticide formulations.

Civil society has criticized the EFSA conclusion referring to the cancerogenic and neurotoxicological potential of GLY [320] and the scientific information and data gaps identified, including the lack of information on the long-term toxicity of one of the representative uses that should have been identified as critical areas of concern by EFSA. However, EFSA's definition of critical areas of concern is clear: if it is established that no safe use can be ensured, if the risk assessment cannot be finalized, or if the criteria laid down in Article 4 of Regulation (EC) 1107/2009 are not met, the EFSA must establish a critical area of concern for one or several endpoints [36]. The state-of-the-art of independent science proves that the harm caused by GLY and its formulations is unacceptable, which

was not made clear in the ECHA and EFSA assessment [321]. EFSAs recent conclusions on GLY recognizes that GLY is toxic to aquatic organisms (category chronic 1—toxic ≤ 0.1 mg l^{-1}, category chronic 2—toxic between 0.1 and 1 mg l^{-1}). In addition, data gaps on aquatic toxicity to aquatic macrophytes and open questions regarding the impact on biodiversity through indirect effects and trophic interactions were identified. These data gaps, the independent studies on the impact of GLY and AMPA on aquatic life, and our findings regarding the current levels of GLY and AMPA contamination of surface waters indicate that the approval criteria are not met.

Despite identified adverse effects of GLY in the scientific literature and the data gaps identified in the EFSA conclusion, the European Commission proposed to reauthorize GLY with certain restrictions. On 28 November 2023, the Commission implementing (EU) Regulation 2023/2660 was published, with allows GLY in the EU for 10 years, with several binding and non-binding restrictions [322]. These include a ban on desiccation with GBH and the requirement to Member States to pay particular attention to the following: (i) uses by non-professional users, (ii) residues that may be present in succeeding crops grown in rotations, (iii) the protection of groundwater in vulnerable areas and of surface waters, (iv) the protection of small herbivorous mammals, (v) the protection of non-target terrestrial and aquatic plants from exposure by spray drift, and (vi) indirect effects on biodiversity via trophic interactions once relevant methods and guidance to identify such effects are agreed at Union level. In addition to this last requirement, the Commission requested that the applicant (companies that applied for the reauthorization of GLY) to submit within three years confirmatory information on the possible indirect effects on biodiversity through trophic interactions. The Commission also proposed maximum application rates, which may only be exceeded if appropriate risk assessments are available. As several national authorities, particularly in smaller Member States, do not have sufficient capacity and resources, it is unlikely that the above listed provisions will be fulfilled. In addition, the status of GLY in the EU, characterized by the recent renewal with additional restrictions, contrasts with the situation in other countries, where there is a complete ban in some countries, cautious use in others, and ongoing legal and public debates that continue to influence policy and perceptions of this widely used herbicide.

Our review is not based on the manufacturers' studies. Some of the studies presented in our review are not included in the EFSA conclusion [320], which has been subject to criticisms, as they indicate the potential harm that GLY/GBHs can cause to aquatic species and ecosystems. Hence, the present review clearly complements EFSAs conclusion and provides novel views. Furthermore, the EFSA conclusion is not really user-friendly as the references are fragmented and lack a single, complete and clear reference section. In addition, the names of authors and publications are often blacked out and not searchable. This review also contains studies that were not included in the EFSA conclusion because they were not considered compliant with Good Laboratory Practice (GLP).

Conclusions

It is widely assumed, especially within regulatory agency circles, that the effects of GLY and its commercial formulations are specific and affect only the target plant species. However, the extensive evidence presented in this review demonstrates that GLY/GBHs can have multiple effects on non-target organisms in aquatic ecosystems. Due to the physiochemical properties of GLY, it can easily enter the aquatic environment. Similarly, multiple effects of GLY and GBHs on terrestrial ecosystems has also evidently been shown [323]. The risks associated with the ecotoxicity of GLY and associated co-formulants in GBHs most likely arise from the higher residue levels resulting from consistent and frequent large-scale application. In general, commercial pesticide formulations consist of AIs and various co-formulants to enhance effectiveness, which includes improving the bioavailability of AIs. These co-formulants have been considered as inactive components with respect to the intended biological effect of commercial pesticide formulations. However, a large and growing number of scientific studies have unequivocally demonstrated the high toxicity of the co-formulants in their own right [31, 32]. This increased combined toxicity of the components present in commercial pesticide formulations has been demonstrated for POEA and many other co-formulants in GBHs [31, 32]. Consequently, POEA has been banned in GBHs under current EU legislation although POEA replacements (e.g., Dodigen 4022, propoxylated quaternary ammonium surfactant) purported as safe alternatives have also proven to be toxic [33, 324]. Therefore, co-formulants cannot be considered inert or inactive ingredients.

The occurrence of residues of GBHs in surface waters is now a globally observed phenomenon. There is a substantial quantity of scientific data available on the acute toxicity of GLY. However, it is difficult to extrapolate and compare the results because the sensitivity of the test organisms, the test conditions, and the composition of the GBHs vary, even if they have the same trade name. Although GLY may be less acutely toxic compared to other herbicidal AIs, unintended adverse outcomes from GLY exposure have been demonstrated in numerous studies on a wide range of aquatic organisms, including

aquatic microorganisms, zooplankton, mollusks, and higher order aquatic plants, fish and amphibians.

One of the fundamental mechanisms underlying these negative effects on the health of various organisms is the induction of oxidative stress, and metabolic and endocrine disruption, which in some cases results in DNA damage [106, 107, 117, 203, 214, 215, 281, 288, 325]. These effects lead to various changes in physiological processes. According to the results of research studies, the tested behavioral endpoints should also be considered during the ERA of pesticides including GBHs. Behavior, as a sublethal endpoint measurement, provides a particularly sensitive and early indication of biotic disruptions and damage compared to severe physiological and mortality-based endpoints [326–329]. The exceptionally high use of GLY has exceeded 800 thousand tons per year since 2014 [39], with current estimates suggesting that it has now exceeded one megaton per year worldwide. Even at a conservative estimate, this amount of GLY is equivalent to three times the amount of phosphorus fertilizer applied annually, in terms of phosphorus content. If GLY is washed into standing water bodies, it can therefore significantly contribute to eutrophication. Currently GLY is the leading pesticide in the market and its use is projected to increase 4.5-fold between 2022 and 2029. This extremely high rate of usage poses a substantial environmental burden resulting in increased exposure and risks to non-target organisms.

Another important issue to address is the consequences of evaluated levels of AMPA, the primary GLY degradation product, in relation to GLY residues found in various water matrices, including surface and drinking water. However, it should be noted that AMPA can be formed not only by the degradation of GLY, but also by its use as a water softener. In the EU, AMPA is not considered a significant metabolite to be taken into account when evaluating the parametric values for GLY in drinking water (0.1 ng ml^{-1}) established in the European Drinking Water Directive (EU) 2020/2184 for pesticide active substances and their relevant metabolites. Nevertheless, some nations such as Denmark, Hungary and France apply a limit value of 0.1 µg l^{-1} for AMPA in drinking water as is the case for pesticide AIs. There is currently no environmental quality standard (EQS) for either GLY or AMPA at the EU level. In a recent proposal, the European Commission revised the list of priority substances for surface water and included an extremely high EQS value for GLY, which would allow a higher level of contamination compared to drinking water safety standards. The same proposal included a threshold of 0.5 µg l^{-1} (AA-EQS—annual average of environmental quality standard) for the combined concentration of pesticide AIs or relevant metabolites, and degradation and reaction products. At the time of writing of this review, the European Commission and EU Member States have not yet determined whether metabolites such as AMPA, which evidently pose a risk to the aquatic environment, will be included in this threshold limit, nor have final EQS values been set by EU policy makers. In 2023, the European Parliament voted on a more ambitious AA-EQS of 0.1 µg l^{-1} for inland surface waters, which is under discussion in the European Council.

Numerous studies assessed in this review indicated that AMPA can have equal and sometimes even stronger detrimental effects compared to GLY in given life stages of aquatic organisms including microorganisms [101], algae and aquatic plants [148, 154], echinoderms and mollusks [196, 199, 201, 330] and fish [234, 246, 255, 258]. AMPA is more persistent in the environment, and EFSAs conclusion of 2023 state that the toxicological profile of AMPA is similar to the toxicity of GLY [320, 331]. Therefore, both AMPA and GLY concentrations should be considered when setting the limit for drinking water. In this regard, the fact that AMPA as a residue may originate from other industrial uses rather than the metabolism of GLY is ecotoxicologically irrelevant. Both GLY and AMPA pose a risk to the aquatic environment, and GLY is already classified as being toxic to aquatic life with long-lasting effects (Aquatic Chronic 2; H411). However, certain studies [81, 84] would justify a more stringent classification.

The combination of GLY and co-formulants often leads to additive or synergistic effects. Furthermore, GLY, GBHs, and even the co-formulants can induce a wide range of lethal or sublethal ecotoxicological outcomes as demonstrated in numerous non-target aquatic organisms even at very low concentrations of exposure (Fig. 1) [198, 201, 216]. Aquatic organisms are highly exposed to aquatic pollutants, and their direct contact with these xenobiotics in water is unavoidable. Therefore, routine monitoring of their exposure is necessary, and the current aquatic toxicity classification of GLY and GBHs should be re-evaluated. The toxicity of GLY in the aquatic environment varies significantly among different species in all taxa and is influenced by exposure conditions such as timing, duration, and extent [74]. Recently, the toxic effects of GLY on amphibians have gained attention in research, indicating that amphibians are particularly susceptible to the effects of GBHs compared to other vertebrates due to their specific lifestyle, which includes both aquatic and terrestrial environments during different life stages [273].

This review presents the results of scientific research that examines the aquatic ecotoxicity of GLY and its commercial formulations as well as the co-formulants present in GBHs. Our review is not based on studies

conducted by the manufacturers. Some of the presented results are not included in the EFSA conclusion published in July 2023 [320]. The observed adverse effects have been demonstrated using a wide variety of endpoints, methods and thresholds to assess the exposure and potential outcomes of the tested substances. It can be concluded that we do not fully know the exact unintended effects of GLY on aquatic non-target organisms and ecosystems even after several decades of GBH use. One of the main problems hindering ecotoxicological assessment is the lack of knowledge of the exact composition of GBHs, which is withheld on the grounds of confidential business information and, therefore, not published. There is still a great need for studies to evaluate the potential toxic effects of co-formulants in GBHs. The current regulation is based on an ERA performed on the AI or commercial pesticide formulations used only once or a few times on a given crop. This is despite the fact that in standard agricultural practice multiple applications of commercial pesticide formulations are conducted during a cultivation cycle. In addition, the effects of commercial pesticide formulations are evaluated on each group of test organisms separately during ERA with interactions between the different trophic levels of the ecosystem not included in the assessment [332, 333]. Furthermore, ERA does not prescribe in-field risks, although biodiversity conservation must be supported to ensure important ecosystem services [333]. The consequences of decades of multiple uses GBHs are not assessed.

In summary, this review has identified important knowledge gaps for a systematic and comprehensive assessment of the aquatic ecotoxicity of GLY and GBH. Therefore, we recommend that the current ERAs be updated to include the following non-exhaustive list of issues:

- Supplement the predominantly short-term, single-species aquatic toxicity testing of GLY and GBH with a focus on aquatic primary producers, invertebrates, or vertebrate (such as fish and amphibians) with multispecies and trophic interactions and indirect effects on aquatic food webs and surrounding landscape.
- At a minimum, include amphibians and reptiles in ERA species lists, as they are among the most threatened species on Earth.
- Investigate the contribution of all ingredients of a GBH, including the various GLY AIs, co-formulants, and other contaminants such as heavy metals [18].
- Evaluate effects on the composition and function of aquatic microbiota inhibited by GLY-effects on their shikimate metabolic pathway.
- Conduct systematic long-term monitoring studies on the effects of high and low chronic exposure in aquatic species with different generation times.
- Evaluate interactions with other contaminants in freshwater and marine ecosystems such as agrochemicals, antibiotics, other chemicals, nutrients, microplastics, light pollution, parasites and climate change factors.
- Explore the impacts of GLY and GBHs on aquatic biodiversity, the consequences of biofilms on food quality at higher trophic levels, and other indirect bottom-up and top-down effects [333].

Some of these knowledge gaps are similar to those previously noted in our review on terrestrial ecotoxicity of GLY and GBHs [323] and also highlighted in the last EFSA Conclusion [320]. Apparently, government regulatory agencies have neglected the ecologically relevant extent of aquatic ecotoxicity in the ERA of GLY and GBHs for decades. Given the serious non-target effects on aquatic ecosystems already identified, and before these serious knowledge gaps are adequately addressed in the ERAs, the precautionary principle enshrined in EU law would actually recommend that GLY/GBHs be withdrawn from the EU market. The current environmental risk assessments and regulatory measures for GLY/GBHs are clearly inadequate to protect aquatic ecosystems and biodiversity.

GBHs mentioned in this review

Aria; Biocarb; C-K Yuyos; Clinic; Credit; Enviro; Factor 540R; Faena; Forceup; Glifoglex; Glifosato II Atanor; GLY-4 Plus; Glyphogan; Glyphogan Classic; Infosato; Kilo Max; Medallon Premium; Orium; Roundup; Roundup Active; Roundup Allées et Terrasses; Roundup Classic; Roundup Express; Roundup Flex; Roundup Full II; Roundup Gran; Roundup Innovert; Roundup LB Plus; Roundup Max; Roundup Power 2.0; Roundup PowerFlex; Roundup Original; Roundup Original MAX; Roundup Original DI; Roundup SL; Roundup Star; Roundup Transorb; Roundup Ultra 360 SL; Roundup UltraMax; Roundup WeatherMax; Roundup Weed & Grass Killer; Sulfosato Touchdown; Sumin Atut; Taifun Forte; Viaglif Jardin; VisionMax.

Abbreviations

AA-EQS	Annual average of environmental quality standard
AChE	Acetylcholinesterase
a.e.	Acid equivalent
AI	Active ingredient
AMPA	Aminomethylphosphonic acid
APG	Alkyl polyglucoside
chl-a	Chlorophyll-a
DNA	Deoxyribonucleic acid
EC_{50}	50% Effective concentration

ECHA	European Chemical Agency
EFSA	European Food Safety Authority
EPS	Extracellular polymeric substances
EQS	Environmental quality standard
ERA	Environmental risk assessment
EU	European Union
GBH	Glyphosate-based herbicide
GDI	Genetic damage index
GGT	γ-Glutamyltransferase
GLY	Glyphosate
GLY-IPA	Glyphosate-isopropylammonium salt
GST	Glutathione-*S*-transferase
GT	Glyphosate-tolerant
GM	Genetically modified
IC_{50}	50% Inhibitory concentration
LC_{50}	50% Lethal concentration
ppm	Parts per million
MXR	Multixenobiotic resistance
POEA	A mixture of polyethoxylated tallow amines
ROS	Reactive oxygen species
RTG-2	Rainbow trout gonad-2
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive species

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Author contributions
SzK: conceptualization; writing—original draft; review and editing. GS: conceptualization; writing—original draft. MO: writing—original draft. ET: writing—original draft. RM: supervision; writing—review and editing. MNA: supervision; writing—review and editing. JGZ: supervision; writing—review and editing. ASz: supervision; writing—review and editing. All authors read and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

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R.M. has served as a consultant on glyphosate risk assessment issues as part of litigation in the United States over glyphosate health effects. The other authors declare that they have no competing interests.

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