

## Herbicides - Properties, Synthesis and Control of Weeds

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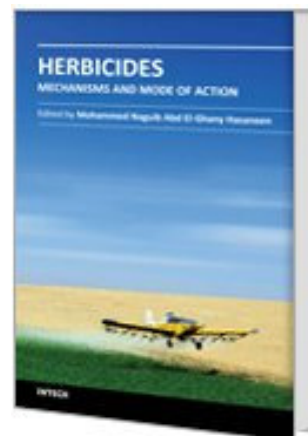
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This book is divided into two sections namely: synthesis and properties of herbicides and herbicidal control of weeds. Chapters 1 to 11 deal with the study of different synthetic pathways of certain herbicides and the physical and chemical properties of other synthesized herbicides. The other 14 chapters (12-25) discussed the different methods by which each herbicide controls specific weed population. The overall purpose of the book, is to show properties and characterization of herbicides, the physical and chemical properties of selected types of herbicides, and the influence of certain herbicides on soil physical and chemical properties on microflora. In addition, an evaluation of the degree of contamination of either soils and/or crops by herbicides is discussed alongside an investigation into the performance and photochemistry of herbicides and the fate of excess herbicides in soils and field crops.

### Chapter 14

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# Forty Years with Glyphosate

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## 1. Introduction

If one were to pick the most notified pesticide of the turn of the millennium, the choice would most likely be glyphosate. Although DDT remains to be the all-time star in the Hall of Fame of pesticides, the second most admitted pesticide active ingredient must be the phosphonomethylglycine type compound of Monsanto Company, glyphosate.

Indeed, the two boasted pesticides show certain similarities in their history of discovery and fate. Both were synthesised first several decades prior to the discovery of their pesticide action. DDT and glyphosate were first described as chemical compounds 65 and 21 years before their discovery as pesticides, respectively. Both fulfilled extensive market need, therefore, both burst into mass application right after the discovery of their insecticide/herbicide activity. They both were, to some extent, connected to wars: a great part of the use of DDT was (and remains to be) hygienic, particularly after World War II, but also the Vietnam War; while glyphosate plays an eminent role in the “drug war” (Plan Colombia) as a defoliant of marijuana fields in Mexico and South America. And last, not least, ecologically unfavourable characteristics of both was applauded as advantageous: the persistence of DDT had been seen initially as a benefit of long lasting activity, and the zwitterionic structure and consequent outstanding water solubility of glyphosate, unusual among pesticides, also used to be praised, before the environmental or ecotoxicological disadvantages of these characteristics were understood.

Yet there are marked differences as well between these two prominent pesticide active ingredients. Meanwhile the career of DDT lasted a little over three decades until becoming banned (mostly) worldwide, the history of glyphosate has gone beyond that by now, since the discovery of its herbicidal action (Baird et al., 1971). And while DDT is the only Nobel prize laureate pesticide, glyphosate was the “first billion dollar product” of the pesticide industry (Franz et al., 1997). Moreover, meanwhile the course of DDT was rather simple: rapid rise into mass utilisation, discovery of environmental persistence, development of pest resistance, loss of efficacy, and subsequent ban; the history of glyphosate is far more diverse: its business success progressed uncumbered, receiving two major boosts. First, the patent protection of glyphosate preparations was renewed in the US in 1991 for another decade on the basis of application advantages due to formulation novelties, and second, its sales were further strengthened outside Europe with the spread of glyphosate-tolerant (GT) genetically modified (GM) crops. This market success has been limited significantly neither

by the recognition of the water-polluting feature of the parent compound, nor by the emerging weed resistance worldwide.

It is not a simple task to predict whether glyphosate continues to rise in the near future, or its application will be abating. To facilitate better assessment of these two possibilities, the present work attempts to provide a summary of the utility and the environmental health problems of glyphosate applications.

## 2. Glyphosate and its biochemistry

### 2.1 The discovery of glyphosate

The molecule *N*-(phosphonomethyl)glycine was first synthesised in 1950 by a researcher of the small Swiss pharmaceutical firm Cilag, Henri Martin (Franz et al. 1997). Yet, showing no pharmaceutical perspective, the compound has not been investigated any further. A decade later through the acquisition of the company, it was transferred to the distributor of laboratory research chemicals, Aldrich Chemical Co., along with research samples of Cilag. This is how it came to the attention of Monsanto Company (St. Louis, MO) in the course of its research to develop phosphonic acid type water-softening agents, through testing over 100 chemical substances related to aminomethylphosphonic acid (AMPA). Monsanto later extended the study of these compounds to herbicide activity testing, and observed their potential against perennial weeds (Dill et al., 2010). *N*-(phosphonomethyl)glycine (later termed glyphosate) was first re-synthesised and tested by Monsanto in 1970. Its herbicidal effect was described by Baird and co-workers in 1971, the subsequent patent (US 3799758), followed by numerous others, was claimed and obtained by Monsanto, and was introduced as a herbicide product Roundup® (formulation of the isopropylamine salt of glyphosate with a surfactant). Upon its introduction in the mid seventies, glyphosate jumped to a leading position on the pesticide market, became the most marketed herbicide active ingredient by the nineties, and more or less holds that position ever since. A great change came about, when the original patent protection expired in many parts of the world outside the United States in 1991. As a result, an almost immediate price decline occurred (by 30% in one year, 40% in two years and about 50% in two decades (Cox, 1998). Upon the expiration of the patent protection also in the United States in 2000, sales of generic preparations intensively expanded (main international producers include Dow, Syngenta, NuFarm, etc.), but the leading preparation producer remained Monsanto (Duke & Powles, 2008).

The current situation of the international active ingredient producers shows a rather different picture. Recently, Chinese chemical factories (e.g., Zhejiang Wynca Chemical Co., Zeijang Jinfanda Biochemical Co. and Hubei Xingta Chemical Group., Nantong Jiangshan Agrochemical and Chemical Co., Sichuan Fuhua Agricultural Investment Group, Jiangsu Yangnong Chemical Group, Jiangshu Good Harvest-Welen, etc.) gained leading parts of this business. At present, the global glyphosate production capacity is 1.1 million tonnes, while the global demand is only 0.5 million tonnes. The overall glyphosate production capacity of Chinese companies rose from 323,400 tonnes in 2007 to 835,900 tonnes in 2010, by a compounded annual growth rate of 37 percent (Yin, 2011). China has enough glyphosate capacity to satisfy the global demand even if all other glyphosate manufacturers cease production. The domestic demand of China is only 30-40 thousand tonnes, about 0.3 million tonnes of glyphosate is produced for export. Presently Chinese glyphosate production

facilities have been suspended being limited by the market demand. Extended use of GT plants in the World would help on this problem, even if Europe is hesitant to allow commercial cultivation of this kind of GM plants. The overall situation has led to continuously decreasing glyphosate prices on the World market, and has significant effects on dispread of GT plants.

## 2.2 Mode of action

Glyphosate is a phosphonomethyl derivative of the amino acid glycine. It is an amphoteric chemical substance containing a basic secondary amino function in the middle of the molecule and monobasic (carboxylic) and dibasic (phosphonic) acidic sites at both ends (Fig. 1). Containing both hydrogen cation ( $H^+$ ) donor (acidic) and acceptor (basic) functional groups, it can form cationic and anionic sites within the small molecule, the dissociation constants ( $pK_a$ ) of these three functional groups are 10.9, 5.9 and 2.3, and therefore, similarly to amino acids, glyphosate can form a zwitterionic structure (Knuuttila & Knuuttila, 1979). This is reflected in excellent water solubility (11.6 g/l at 25 °C). Consequently, its lipophilicity is very low ( $\log P < -3.2$  at 20 °C, pH 2-5), and is insoluble in organic solvents e.g., ethanol, acetone or xylene (Tomlin, 2000). To further increase its already good water solubility it is often formulated in form of its ammonium, isopropylammonium, potassium, sodium or trimethylsulphonium (trimesium) salts. The order of water solubility is glyphosate  $\ll$  ammonium salt  $<$  sodium salt  $<$  potassium salt  $<$  isopropylammonium salt  $<$  trimesium salt, the solubility of the trimesium salt being two orders of magnitude higher than that of glyphosate.

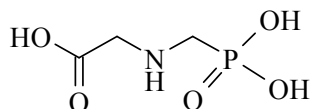


Fig. 1. The chemical structure of *N*-(phosphonomethyl)glycine, glyphosate, containing a basic function (amine) in the middle of the molecule and two acidic moieties (carboxylic and phosphonic acids) at both ends.

It has been known since the early seventies that glyphosate acts by inhibiting aromatic amino acid biosynthesis in plants (Jaworski, 1972; Amrhein et al., 1980), and elaborate research has revealed that the responsible mechanism is blocking a key step in the so-called shikimate pathway (Herman & Weaver, 1999), responsible for the synthesis of aromatic amino acids and critical plant metabolites. Glyphosate exerts this effect by inhibiting the activity of the enzyme 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) catalyzing the transformation of phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) (Amrhein et al., 1980). This metabolic pathway exists in plants, fungi, and bacteria, but not in animals (Kishore & Shah 1988). Although higher order living organisms lack this metabolic route, therefore, are not expected to be directly affected by this herbicide, the environmental consequences of the widespread use of glyphosate have been reported (Cox, 2000; Santillo et al., 1989).

Being an amino acid (glycine) derivative itself, glyphosate inhibits the formation of the main intermediate, by binding as an analogue of the substrate PEP to its catalytic site on the enzyme. The inhibition of this catabolic pathway blocks the synthesis of triptophan, phenylalanine and tyrosine, and in consequence, the synthesis of proteins. The lack of the

synthesis of these essential amino acids and the proteins that contain them leads to rapid necrosis of the plant. Because this metabolic pathway is present in all higher order plants, and because the amino acid sequence of the active site of the EPSPS is a very conservative region in higher plants, the herbicidal effect is global among plant species.

Moreover, through its excellent solubility features glyphosate is a systemically active herbicide ingredient. As it is capable to be transported in the plant from the leaves towards the roots, it belongs to the relatively uncommon group of basipetally translocated herbicides (Ashton & Crafts, 1981). Its uptake and translocation is relatively rapid in diverse species (Sprankle et al., 1975).

### 2.3 Transition state analogue theory of enzyme inhibition

A unique feature of the mechanism of the inhibition of EPSPS by glyphosate is that glyphosate is reported to show close similarity in its structure to the tetrahedral phosphoenolpyruvoyl oxonium ion derivative of PEP, formed during its catalytic conversion to S3P, and the adduct formation with EPSPS has been verified by nuclear magnetic resonance spectroscopy (Christensen & Schaefer, 1993). Therefore, it has been proposed that glyphosate exerts its inhibitory activity as transition-state analogue (TSA) of the putative phosphoenolpyruvoyl oxonium ion derivative of PEP from plants (Anton et al., 1983; Steinrücken & Amrhein, 1984; Kishore & Shah, 1988) and bacteria (Du et al., 2000; Arcuri et al., 2004).

The so-called transition state theory has been advanced by Pauling (1948) to explain the mechanism of enzymatic reactions. Enzymes are catalysts therefore they accelerate a reaction without influencing its equilibrium constant. One way to achieve that is to diminish the energy barrier of the reaction by lowering the energy of the transition state, transient, unstable intermediate of the reaction. This may be accomplished through stabilizing the transition state by binding to it as soon as it has occurred, and thus facilitating its formation. This results in the enzymatic effect that lowers the activation energy of the catalyzed reaction. Based on this idea, extremely potent inhibitors can be developed for a given enzymatic reaction if one can synthesize “transition state analogues” or “transition state mimics”: stable chemical compounds resembling the transition state (Wolfenden, 1969). The TSA theory has therefore been successfully applied to the development of various biologically active substances, including insect control agents (Hammock et al., 1988), sulfonyleurea microherbicides (Schloss & Aulabaugh, 1990) or compounds related to glyphosate (Marzabadi et al., 1992; Anderson et al., 1995).

The TSA hypothesis as it applies to the mechanism of the inhibition of EPSPS by glyphosate, became widely accepted as it has been evidenced in numerous studies that glyphosate forms a tight ternary complex with EPSPS (Herman & Weaver, 1999). It is easy to understand, however, that a classical TSA inhibitor would cause irreversible inhibition of the enzyme, competeable (although possibly with a low affinity) by the natural substrate of the enzyme. In later studies, it has been evidenced by biochemist researchers of Monsanto that glyphosate was an inhibitor of EPSPS uncompetitive with EPSP, and therefore, the TSA hypothesis has been reconsidered (Sammons et al., 1995; Schönbrunn et al., 2001; Alibhai & Stallings, 2001; Funke et al., 2006). The effects of glyphosate on aromatic amino acid synthesis in *Escherichia coli* have been attributed to chelation of  $\text{Co}^{2+}$  and  $\text{Mg}^{2+}$  (Roisch & Lingens, 1980), cofactors for enzymes in this pathway. Moreover, it is interesting, that glyphosate does not inhibit the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase

(Samland et al., 1999), structurally and mechanistically closely related EPSPS, and playing a key role in the biosynthesis of UDP-muramic acid.

#### 2.4 Other biochemical effects of glyphosate

Various biochemical interactions of glyphosate, besides its identified mode of action, in plants and microorganisms were summarised by Hoagland and Duke (1982). The authors refer to numerous secondary or more complex indirect effects of glyphosate, and point out that a compound with such a powerful growth retardant effect or strong phytotoxicity will ultimately affect virtually all biochemical processes in the affected cells.

The effects of glyphosate in the plant possibly include influences on the regulation of hormonal processes. Methionine levels are greatly reduced by glyphosate (Duke et al., 1979), which suggests that this herbicide may alter ethylene biosynthesis. Results of Baur (1979) suggest that glyphosate may inhibit auxin transport by increasing ethylene biosynthesis. Glyphosate may also affect the biosynthesis of non-aromatic amino acids. Nilsson (1977) suggested that the build-up of glutamate and glutamine in glyphosate-treated tissue might be due to blocked transamination reactions.

It has been hypothesised that glyphosate lower phenylalanine and tyrosine pools not only by its primary mode of action, but possibly also by induction of phenylalanine ammonia-lyase (PAL) activity. Indeed, pronounced PAL activity has been detected in glyphosate-treated maize and soy (Duke et al., 1979; Cole et al., 1980), yet not by direct effect according to *in vitro* tests. Therefore, although glyphosate has been evidenced to cause profound effects on extractable PAL, substrate(s) and end products, increased PAL activity has been evaluated as a secondary effect (Hoagland & Duke, 1982).

Glyphosate did not appear to cause direct effects on photosynthesis, but its possible effect on chlorophyll biosynthesis has been considered, and its strong inhibitory effect on chlorophyll accumulation has been shown (Kitchen et al., 1981). Experimental result indicated that the effect of glyphosate on chlorophyll may be indirect through photobleaching and/or peroxidation of chlorophyll.

Glyphosate has been shown to significantly affect the membrane transport of cellular contents only at very high concentrations (Brecke & Duke, 1980; Fletcher et al., 1980). Phosphorous uptake was retarded (Brecke & Duke, 1980), but loss of membrane integrity, decrease in energy supply or external ion chelation were excluded as causes. Moreover, uptake of amino acids, nucleotides and glucose were also found to be retarded by glyphosate in isolated cells (Brecke & Duke, 1980). Other studies (Cole et al., 1980; Duke & Hoagland, 1981) found inhibition of amino acid uptake by glyphosate not severe. Glyphosate has been reported to uncouple oxidative phosphorylation in plant (Olorunsogo et al., 1979) and mammalian (Olorunsogo & Bababunmi, 1980) mitochondria, the latter is likely to be due to altered membrane transport processes, as glyphosate was found to enhance proton permeability of mitochondrial membranes in a concentration-dependent manner (Olorunsogo, 1990).

### 3. Pre-emergent application technology of glyphosate

Glyphosate, exerting global herbicidal action, has originally been intended to pre-emergent weed control treatments of field vegetation and weed control of orchards and ruderal areas.

Post-emergent applications are impossible solely with glyphosate-based herbicide formulations due to the phytotoxicity of the compound to the crop as well.

Common first visible phytotoxicity effects of glyphosate include rapid (within 2-10 days upon application) chlorosis, usually followed by necrosis (Suwannamek & Parker, 1975; Putnam, 1976; Campbell et al., 1976; Fernandez & Bayer, 1977; Marriage & Khan, 1978; Segura et al., 1978; Abu-Irmaileh & Jordan, 1978), possibly accompanied with morphological leaf deformities (Marriage & Khan, 1978), root and rhizome damage (Suwannamek & Parker, 1975; Fernandez & Bayer, 1977). Glyphosate accumulation has been reported in the meristems (Haderlie et al., 1978). It is rather surprising that although glyphosate inhibits seedling growth as well, it did not exert significant effect on the germination of various species (Haderlie et al., 1978; Egley & Williams, 1978).

### 3.1 Formulated glyphosate-based herbicides

Glyphosate-based formulations such as Roundup®, Accord® and Touchdown® represent the most common types used for agricultural purposes (Franz et al., 1997). These formulated herbicides can be used for weed control in agricultural practice, including in no-till agriculture to prepare fields before planting, during crop development and after crop harvest; as well as in silvicultural, urban and, lately, aquatic environments. The main herbicide products currently distributed are listed in Table 1. These preparations contain glyphosate as formulated in form of its ammonium (AMM), dimethylammonium (DMA), isopropylammonium (IPA), potassium (K) or trimesium (TRI) salts. The very first formulations containing IPA, sodium and ammonium salts were patented by Monsanto in 1974. A unique form is the trimesium salt of outstanding water solubility, patented by ICI Agrochemicals (later Zeneca Agricultural Products Inc, then Novartis CP, and after 2000 Syngenta) in 1989 (Tomlin, 2000).

As the actual active ingredients of the formulations are salts, differing from each other in the cation(s) and consequently the molecular mass of the salts, active ingredient concentrations are specified as glyphosate equivalent, in other term acid equivalent (a.i.) referring to the free acid form of glyphosate. This provides instant comparability among various formulations. Moreover, the use of a.i. units is common practice in residue analysis of glyphosate as well.

### 3.2 Formulating agents

Formulated glyphosate-based herbicides contain various non-ionic surfactants to facilitate their uptake by the plants (Riechers et al., 1995). These components, as all other pesticide additives and diluents, are assumed to be inert, which as it turns out, is not the case for several such ingredients. The most common surfactant applied in combination with glyphosate is polyethyloxytated tallowamine (POEA), which itself has been found to exert ecotoxicity, also in synergy with glyphosate, causing the formulated herbicide (e.g., Roundup) more toxic than its technical grade active ingredient (Folmar et al., 1979; Atkinson, 1985; Wan et al., 1989; Powell et al., 1991; Giesy et al., 2000; Tsui & Chu, 2003; Marc et al., 2005; Benachour et al., 2007; Benachour & Séralini, 2009).

The apparent synergistic toxic effects of the assumedly inert ingredients with glyphosate triggered a legal case between Monsanto and the New York Attorney General's Office in

Manufacturer	a.i. salt <sup>a</sup>	Product <sup>b</sup>
AAKO B.V.	IPA	Akosate
Agriliance LLC	IPA	Cornerstone
Agro-Chemie Ltd.	IPA	Fozát
Albaugh Inc./ Agri Star	IPA	Aqua Star, Gly Star Original
Astrachem Ltd.	IPA	Tiller
Barclay Chem. Mfg. Ltd.	IPA	Gallup
Calliope S.A.	IPA	Kapazin
Chemical Products Technologies LLC	IPA	ClearOut; ClearOut Plus
Cheminova	IPA	Glyfos; Glyphos X-tra
Control Solutions Inc.	IPA	Spitfire
Crystal Chem. Inter-America	IPA	Glifonox
Dow AgroSciences	IPA	Dominator; Durango; Glyphomax; Glyphomax Plus; GlyPro; Panzer; Ripper; Rodeo; Vantage
	DMA	Durango DMA; Duramax
Drexel Chem. Co.	IPA	Imitator
	K	DupliKator
FarmerSaver.com LLC	IPA	Glyphosate 4
Griffin LLC	IPA	Glyphosate Original
Growmark Inc.	IPA	FS Glyxphosate Plus
Helena Chemical Co.	IPA	Rattler
	IPA + AMM	Showdown
Helm Agro US Inc.	IPA	Glyphosate 41%; Helosate Plus
Loveland Products Inc.	IPA	Mad Dog; Mirage
Makhteshim-Agan	IPA	Eraser, Gladiator; Glyphogan; Hardflex; Herbolex; Taifun
Micro Flo	IPA	Gly-Flo
Monsanto Co.	IPA	Accord; Aquamaster; Azural; Clinic; Gialka; Honcho; Ranger Pro, Roundup Bioforce / Classic / Original / UltraMAX
	K	Roundup Forte / Mega / PowerMAX / WeatherMAX; VisionMAX
Nufarm	IPA	Amega; Credit; Credit Extra
	IPA + MA	Credit Duo
Oxon Italia S.p.A.	AMM	Buggy
Pinus TKI d.d.	IPA	Boom Efekt
Sinon Corporation	IPA	Glyfozat; Total
Syngenta AG	AMM	Medallon Premium
	DMA	Touchdown IQ
	K	Refuge; Touchdown HiTech / Total; Traxion
	TRI	Coloso; Ouragan
Tenkoz Inc.	IPA	Buccaneer
UAP	IPA	Makaze
Universal Crop Protection Alliance LLC	IPA	Gly-4
Winfield Solutions LLC	IPA	Cornerstone

<sup>a</sup> AMM = ammonium; DMA = dimethylamine; IPA = isopropylamine; K = potassium; TRI = trimesium

<sup>b</sup> Formulations containing only glyphosate salts as active ingredient are listed, herbicide combinations are not included

Table 1. Formulated herbicide preparations containing glyphosate as active ingredient.



1996 (Attorney General of the State of New York, 1996). The toxicological basis of the legal claim was that Monsanto inaccurately implied toxicity data of the active ingredient glyphosate on the formulated product Roundup. As a result of the lawsuit, Monsanto was fined, and agreed to drop description of being “environmentally friendly” and “biodegradable” from the advertisements of the herbicide.

Concerns about application safety, triggered by the above studies and findings on teratogenic effects (see 6.3 Teratogenic activity of glyphosate), have brought re-registration of glyphosate and its formulated products in focus in the European Union, as part of the regular pesticide revision process due to take place in 2012. Nonetheless, the EU Commission dismissed these findings, based on a rebuttal by the EU “rapporteur” member state for glyphosate, Germany, provided by the German Federal Office for Consumer Protection and Food Safety (BVL), and postponed the review of glyphosate and 38 other pesticides until 2015 (European Commission, 2010). To protest against such delay in re-evaluation of these 39 pesticides, the Pesticides Action Network Europe and Greenpeace brought a lawsuit against the EU Commission, and the dismissal of the reported teratogenicity data from the official current evaluation has been judged by several researchers as irresponsible act (Antoniou et al., 2011).

#### 4. Post-emergent application technology of glyphosate

A group so far of the highest financial importance within GM crops has been modified to be tolerant to this active ingredient, outstandingly broadening its application possibilities.

##### 4.1 Glyphosate-tolerant crops

Upon pre-emergent applications of the global herbicide glyphosate, the majority of the weeds decays, perishes, and does not get consumed by wild animals. This situation has been changed tremendously by the appearance of GT crops, leading to increasing environmental herbicide loads due to approved post-emergent treatments (2-3 applications in total). Of these crops, the varieties of Monsanto became most publicised, under the trade mark Roundup Ready® (RR), indicating that these plants can be treated with the herbicide preparation of Monsanto, Roundup® containing glyphosate as active ingredient even, after the emergence of the crop seedlings. Similar varieties by Bayer CropScience, Pioneer Hi-Bred and Syngenta AG are termed Gly-Tol™, Optimum® GAT® and Agrisure® GT, respectively. Two strategies have been followed by plant gene technology in the development of GT varieties: either the genes (*cp4 epsps*, *mepsps*, *2mepsps*) of mutant forms of the target enzyme less sensitive to glyphosate or genes (*gat*, *gox*) of enzymes metabolizing glyphosate have been transferred into the GM plant varieties (Table 2). The genetically created tolerance to glyphosate does not alter the mode of action of the compound: the molecular mechanism of glyphosate tolerance has been elucidated (Funke et al., 2006), and the sole mechanism of inhibition remains blocking of the shikimate pathway when applied at very high doses on GT soybean and canola (Nandula et al., 2007).

The first GT crop was RR soybean by Monsanto in 1996, followed by GT cotton, GT maize, GT canola, GT alfalfa and GT sugarbeet (Dill et al., 2008). GT crops allow a new form of technology, post-emergent application of glyphosate. The utilizability of post-emergent applications was systematically tested in 2002 and 2003 in field experiments in the United States (Parker et al., 2005). The extensive study involving GT maize and GT soybean sites at

Variety owner	Crop	Genetical event	Transgene introduced <sup>a</sup>
Bayer CropScience (part of Sanofi-Aventis)	Cotton	<i>GHB614</i>	<i>2mepsps</i>
Monsanto Co.	Cotton	<i>MON 1445</i>	<i>cp4 epsps, nptII, aad</i>
	Cotton	<i>MON 88913</i>	<i>cp4 epsps</i>
	Maize	<i>MON 88017</i>	<i>cp4 epsps, cry3Bb1</i>
	Maize	<i>NK603</i>	<i>cp4 epsps</i>
	Rape	<i>GT 73</i>	<i>cp4 epsps, gox</i>
	Soybean	<i>MON40-3-2</i>	<i>cp4 epsps</i>
	Soybean	<i>MON 87705</i>	<i>cp4 epsps, FAD2-1A, FATB1-A</i>
	Soybean	<i>MON 89788</i>	<i>cp4 epsps</i>
	Sugar-beet <sup>b</sup>	<i>A5-15</i>	<i>cp4 epsps, nptII,</i>
Sugar-beet <sup>c</sup>	<i>H7-1</i>	<i>cp4 epsps</i>	
Pioneer Hi-Bred (part of DuPont)	Maize	<i>DP-98140</i>	<i>Gat4601, als</i>
	Soy	<i>DP-356043</i>	<i>gat4601</i>
Syngenta	Maize	<i>GA21</i>	<i>mepsps</i>

<sup>a</sup> *aad* – gene of *Escherichia coli* origin, encoding resistance against aminoglycoside antibiotics (streptomycin and spectinomycin); *als* – gene (*zm-hra*) of maize origin, enhancing tolerance of ALS inhibiting herbicides (e.g., chlorimuron and thifensulfuron); *cry3Bb1* – gene of *Bacillus thuringiensis* origin, encoding Cry3 toxin; *FAD2-1A* – gene of soy origin, encoding fatty acid desaturase enzyme, silencing of which enhances the proportion of monounsaturated fatty acids; *FATB1-A* – gene of soy origin, encoding medium-chain fatty acid thioesterase, silencing of which reduces the proportion of saturated fatty acids; *cp4 epsps* – *epsps* gene of *Agrobacterium* sp.; *mepsps* – *epsps* gene of maize origin; *2mepsps* – double mutated *epsps* gene of Mexican black, sweet maize origin; *gat4601* – gene of *Bacillus licheniformis* origin, encoding glyphosate acetyltransferase enzyme; *gox* – gene of *Ochrobactrum anthropi* origin, encoding glyphosate oxidase enzyme; *nptII* – gene of *Escherichia coli* K12 origin, encoding neomycin phosphotransferase, causing neomycin and kanamycin resistance.

<sup>b</sup> together with Danisco Seeds and DLF Trifolium as variety owners

<sup>c</sup> together with KWS Saat Ag. as variety owners

Table 2. Glyphosate tolerant crop variety groups under registration process in the European Union.

seven locations, as well as regular or directed post-emergent applications of 10 formulated glyphosate preparations (ClearOut 41 Plus<sup>TM</sup>, Gly Star<sup>TM</sup>, Glyfos<sup>®</sup>, Glyfos<sup>®</sup> X-tra, Glyphomax<sup>TM</sup>, Roundup Original<sup>TM</sup>, Roundup UltraMAX<sup>®</sup>, Roundup WeatherMAX<sup>TM</sup>, Touchdown<sup>®</sup> and Touchdown Total<sup>TM</sup>) containing isopropylamine or potassium salts of glyphosate found no herbicide efficacy or produce quality differences, no phytotoxicity to maize and medium phytotoxicity to cotton at high doses in some instances, and therefore proposed post-emergent glyphosate applications. As a result, the use of glyphosate has expanded almost 20-fold by 2007 in the United States (Pérez et al., 2011).

Another impact of GT crops on agricultural practices is the spread of no-till agriculture. As the crop tolerates the active ingredient, intensive herbicide treatments are possible to be carried out, instead of former tillage practices, to eradicate vegetation in the field. This has greatly increased herbicide use and consequent chemical pressure on the environment. No-till practice is particularly common in GT crop cultivating areas in South America, including Brazil, Argentina, Paraguay and Uruguay (Altieri & Pengue, 2006).

An interesting detail is that in parallel to industrial development of GT crops, illegal genetic modification projects are also being carried out to achieve “crops” that are resistant to

glyphosate e.g, a new marijuana (*Canabis* sp.) hybrid that can be cultivated all year and cannot be controled with herbicides (Anonymous, 2006). The GT marijuana hybrid, first appeared in Mexico in 2004, allows 8-9-times higher yields than “conventional” varieties, and became the plant of choice for drug traffickers in Michoacan.

## 4.2 The effect of glyphosate-tolerant crops on glyphosate residues

As a result of the combined effect of the expiration of the patent protection of glyphosate (in 2000 in the United States) and the spread of cultivation of GT GM crops (since 1996 in the United States), the use of glyphosate products is again increasing (Woodburn, 2000). Besides GT GM crops, energy crop cultivation is also an and emerging source of glyphosate contamination (Love et al., 2011). Moreover, due to the modified metabolic pool in the GT GM crops, residues of the systemic glyphosate active ingredient are expected to occur in the surviving plants. In case of EPSP-mutant (RR and Agrisure GT) varieties, the residue composition is expected to be similar to those seen at regular glyphosate applications, while in the case of the boosted glyphosate metabolizing (regardless whether *epsps* or *gox* transgene based) varieties, increased amounts of *N*-acetylglyphosate (NAG) (Optimum GAT variety) or aminomethylphosphonic acid (AMPA) (RR and Agrisure GT varieties) are expected in the plants. In turn, residue patterns not yet seen in food and feed are to be expected. Summarizing the results of their studies in Argentina between 1997 and 1999, Arregui and co-workers (2004) reported glyphosate residue levels after 2-3 glyphosate applications as high as 0.3-5.2 mg glyphosate/kg and 0.3-5.7 mg AMPA/kg in the leaves and stem of RR soy during harvest, and 0.1-1.8 mg glyphosate/kg and 0.4-0.9 mg AMPA/kg in the produce. In turn, glyphosate occurred as surface water, soil and sediment contaminant in a GM soybean cultivating area in Argentina (Peruzzo et al., 2008).

## 5. The environmental fate of glyphosate

### 5.1 Residue analysis of glyphosate

Present analytical methods developed for the detection of glyphosate are mostly based on separation by liquid chromatography (LC), as previous methods utilizing gas chromatography (GC) have become of much lesser importance than they used to be (Stalikas & Konidari, 2001). The main obstacle in the GC detection of glyphosate and its main metabolite AMPA is the polaric and zwitterionic structure of these compounds, which required laborious sample preparation steps prior to instrumental analysis. The earliest method accredited for authoritative analytical determination of glyphosate (US FDA, 1977) employed aqueous extraction, anion and cation exchange purification, *N*-acetylation derivatisation with trifluoroacetic acid and trifluoroacetic anhydride, and subsequent methylation of both the carboxylic acid and phosphonic acid moieties on the parent compound, followed by GC analysis with phosphorous-specific flame ionisation detection. Recoveries above 70% were achieved by the method in plant samples, the limit of detection (LOD) was 0.05 mg/kg. The basis of the protocol was the GC-MS derivatisation method developed by Monsanto (Rueppel et al., 1976). A later method by Alferness and Iwata (1994) also employs aqueous extraction, followed by washing with dichloromethane/chloroform, purification on cation exchange column, derivatisation to trifluoroacetate and heptafluorobutyl ester, followed by GC analysis with mass spectrometry (MS) detection, and a similar methods have also been developed (Tsunoda, 1993; Natangelo et al., 1993;

Royer et al., 2000; Hudzin et al., 2002). Validated LC methods also resulting in similar analytical parameters (Cowell et al., 1986; Winfield et al., 1990; DFG, 1992) utilise washing with chloroform and hydrochloric acid, purification on ion exchange column, and upon neutralisation and derivatisation with *o*-phthalic aldehyde and mercaptoethanol, determination by high performance liquid chromatography (HPLC) with fluorescence detection. Yet the LOD of the official method (Method 547) established by the U.S. Environmental Protection Agency is as high as 6 µg/l in reagent water and 9 µg/l in surface water (Winfield et al., 1990). Ninhydrin or 9-methylfluorenyl chloroformate have also been applied as derivatising agents (Wigfield & Lanquette, 1991; Sancho et al., 1996; Nedelkoska & Low, 2004; Peruzzo et al., 2008). More recent LC procedures with somewhat simplified sample preparation steps offer rapid and more economic analytical methods than GC procedures always requiring complex, often several step derivatisation. As a result, GC methods remain being used solely due to their analytical parameters, including sensitivity. Nonetheless, LODs of LC and ion chromatographic methods were achieved to be lowered (Mallat & Barceló, 1998; Vreeken, 1998; Bauer et al., 1999; Grey et al., 2001; Patsias et al., 2001; Lee et al., 2002a; Nedelkoska & Low, 2004; Ibáñez et al., 2006; Laitinen et al., 2006; Hanke et al., 2008; Popp et al., 2008) to meet the strictening maximal residue levels (MRLs) in environmental and health regulations. The most recent LC-MS methods using electrospray ionisation (Granby et al., 2003; Martins-Júnior et al., 2011) easily meet the MRL by the EU for given pesticide residues in drinking water, 0.1 µg/l, but the instrumentation demand of these methods is substantial.

Among novel innovative analytical methods for the detection of glyphosate, mostly capillary electrophoresis (CE) and immunoanalytical methods are to be mentioned. Initial drawbacks of the CE methods included relatively high LOD and the need for derivatisation or external fluorescent labeling (Cikalo et al., 1996; You et al., 2003; Kodama et al., 2008), later solved by coupling CE with MS (Goodwin et al., 2003) and microextraction techniques (Hsu and Whang 2009; See et al., 2010). Among various immunoanalytical techniques, enzyme-linked immunosorbent assays (ELISAs) gained the highest utility. While in the early nineties we considered yet that effective antibodies are not produced against glyphosate and similar zwitterionic compounds due to their low immunogenicity (Hammock et al., 1990), difficulties in immunisation have been overcome within a decade, and sensitive ELISAs, also employing derivatisation, were developed (Clegg et al., 1999; Lee et al., 2002b; Rubio et al., 2003; Selvi et al., 2011), proven to be of great utility in environmental analytical studies for glyphosate (Mörtl et al., 2010; Kantiani et al., 2011). On the basis of the immunoassay principle, sensors using glyphosate-sensitive antibodies (González-Martínez et al., 2005) or molecularly imprinted polymers (MIPs) (Zhao et al., 2011) were also developed.

## 5.2 Glyphosate and its decomposition products

Decomposition of glyphosate takes place mostly by two processes: decarboxylation or dephosphorylation, and the corresponding intermediate metabolites are AMPA or glycine, respectively. The first pathway is catalyzed by oxidoreductases, the second by C-P lyases cleaving the carbon-phosphorous bond. Both pathways occur in environmental matrices (water, soil) and plants, but the main metabolite in all cases is AMPA (Fig. 2). The environmental fate, behaviour and analysis of both AMPA and glyphosate has received considerable attention (Stalikas & Konidari, 2001).

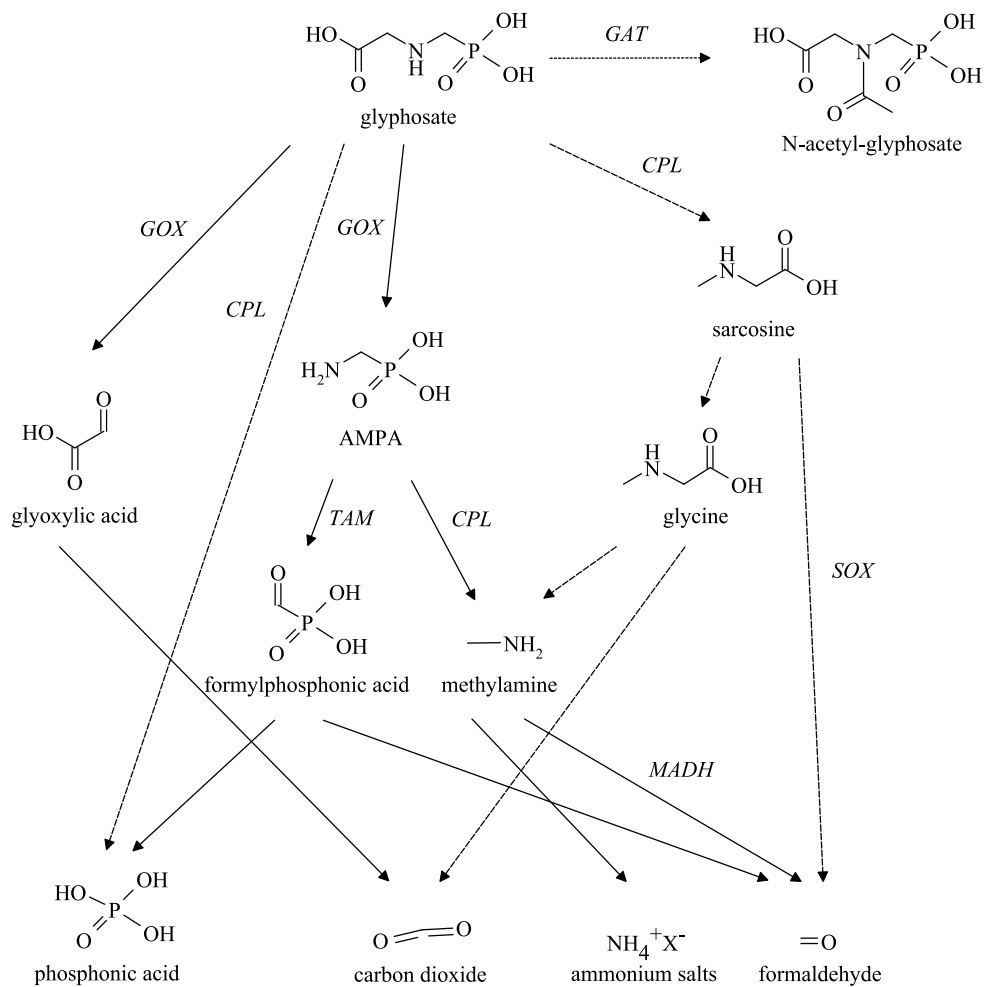


Fig. 2. Possible fate of glyphosate by various metabolizing pathways. Oxidative decomposition (*solid arrows*), non-hydrolytic decomposition (*dashed arrows*), inactivation in plants (*dotted arrow*). Processing enzymes (*Italic letters*) - *GOX*: glyphosate oxidoreductase, *GAT*: glyphosate *N*-acetyltransferase, *CPL*: C-P-liase, *SOX*: sarcosin oxidase, *TAM*: transaminase, *MADH*: methylamine dehydrogenase.

AMPA has been reported to be rapidly formed microbiologically, but not by chemical action, in water and in various loam soils (Drummer silty clay loam, Norfolk sandy loam, Ray silt loam, Lithonia sandy loam) (Rueppel et al., 1977; Aizawa, 1982; Mallat & Barceló, 1998), and was shown to be degraded subsequently completely to carbon dioxide (Sprankle et al., 1975; Rueppel et al., 1977; Moshier & Penner, 1978). Chemical processes of degradation are ineffective because of the presence of a highly stable carbon-phosphorus bond in the compound (Gimsing et al., 2004). Which pathway is predominant in the microbial degradation depends on bacterial species. The first (AMPA) pathway is

commonly seen in mixed soil bacterial cultures (Rueppel et al., 1977) and certain *Flavobacterium* sp. The glycine pathway is characteristic to certain *Pseudomonas* and *Arthrobacter* sp. strains (Jacob et al., 1988). AMPA is further metabolised, providing phosphorus for growth, although the amount eliminated is typically set by the phosphorus requirement of the bacterium in question. Sarcosine and glycine are other possible main degradation products in soils (Rueppel et al., 1977).

As for decomposition in water or soil, the stability of glyphosate depends of a number of parameters. It strongly interacts with soil components by forming tight complexes with numerous metal ions in solution and by being adsorbed on soil particles, including clay minerals. Adsorption is strongly influenced by cations associated with the soil (Carlisle & Trevors, 1988), and it is mainly the phosphonic acid moiety that participates in the process, therefore, phosphate competes with glyphosate in soil adsorption (Gimsing & dos Santos, 2005). As a result of its adsorption on clay particles and organic matter present in the soil, upon application glyphosate remains unchanged in the soil for varying lengths of time (Penaloza-Vazquez et al., 1995). Adsorption of chelating agents by surfaces has been shown to decrease biodegradability. It can be expected that phosphonates with their higher affinity to surfaces are much slower degraded in a heterogeneous compared to a homogeneous system, as seen for glyphosate (Zaranyika & Nyandoro, 1993).

Therefore, differences have been observed between half-lives ( $DT_{50}$ ) of glyphosate determined in laboratory or field studies. Half-lives were found quite favourable in laboratory, 91 days in water and 47 days in soil. Nonetheless, half-life of the parent compound ranged between a few days to several months or even a year in field studies, depending on soil composition. A reason of such delayed decomposition is partly binding to the soil matrix, through which glyphosate adsorbed on soil particles can form complexes with metal (Al, Fe, Mn, Zn) ions (Vereecken, 2005). By the increased solubility of its various alkali metal, ammonium or trimesium salts, the active ingredient can leach into deeper soil layers, in spite of its rapid decomposition and strong complex formation capability under certain conditions (Vereecken, 2005). Its primary metabolite AMPA is more mobile in soil than the parent compound (Duke & Powles, 2008).

Moreover, decomposition dynamics of glyphosate is greatly dependent on the microbial activity of soil, with mostly *Pseudomonas* species as most important microbial components (Borggaard & Gimsing, 2008). If microbial activity is elevated, glyphosate is degraded with reported laboratory and field half-life of < 25 days and 47 days, respectively (Ahrens, 1994). Moreover, glyphosate itself affects the survival of soil microorganisms (Carlisle & Trevors, 1988; Krzysko-Lupicka & Sudol, 2008). Studies of glyphosate degrading bacteria have involved selection for, and isolation of pure bacterial strains with enhanced or novel detoxification capabilities for potential uses in biotechnology industry and biodegradation of polluted soils and water. Microorganisms known for their ability to degrade glyphosate in soil and water include *Pseudomonas* sp. strain LBr (Jacob et al., 1988), *Pseudomonas fluorescens* (Zboinska et al., 1992), *Arthrobacter atrocyaneus* (Pipke et al., 1988) and *Flavobacterium* sp. (Balthazor & Hallas, 1986). Soil microbial activity, however, depends on a number of additional parameters, including soil temperature, abundance of air and water, and a number of not yet defined factors, creating rather variable conditions for the decomposition of glyphosate (Stenrød et al., 2005; 2006). Other studies have also shown that soil sorption and degradation of glyphosate exhibit great variation depending on soil composition and properties (de Jonge et al., 2001; Gimsing et al., 2004a, 2004b; Mamy et al.,

2005; Sørensen et al., 2006; Gimsing et al., 2007). Laitinen and co-workers (2006; 2008) reported that phosphorous content in the soil affects the environmental behaviour of glyphosate e.g., its absorbance on soil particles, and its occurrence in surface waters. Weaver and co-workers (2007) claim that its effects on soil microbial communities are short and transient, and that decomposition characteristics of glyphosate do not change significantly in lower soil layers in Mississippi with various tilling methods (Zablotowicz et al., 2009). Outstandingly different result were obtained in an environmental analytical study carried out in Finland, who detected 19% of the applied glyphosate undecomposed and 48% in form of AMPA 20 months after application in Northern European soils of low phosphorous content (Laitinen et al., 2009). This also sheds a light on the high reported glyphosate contamination levels in Scandinavian surface waters (Ludvigsen & Lode, 2001a; 2001b). The phosphorous content of the soil may also play a key role in the low decomposition rate seen through its effect on microbial communities, as soil phosphorous has been shown to be able to stimulate decomposition of glyphosate (Borggaard & Gimsing, 2008). An interesting interaction observed is that persistence of glyphosate significantly increased in soils treated with Cry toxins of *Bacillus thuringiensis* subsp. *kurstaki*, while a similar effect was not seen when soils were treated with purified Cry1Ac toxin (Accinelli et al., 2004; 2006). Therefore, it is worthwhile reconsidering the fate of glyphosate in soils, including sorption, degradation and leachability.

Due to its strong sorption and relatively fast degradation in soil, glyphosate has been claimed to cause very limited risk of leaching to groundwater (Giesy et al., 2000; Busse et al., 2001; Vereecken, 2005; Cox & Surgan, 2006). Yet, other investigations indicates possible leaching and toxicity problems with its use (Veiga et al., 2001, Strange-Hansen, 2004; Kjær, 2005; Landry et al., 2005; Relyea, 2005b; Torstensson et al., 2005; Siimes et al., 2006) and consequent effects on aquatic microbial communities (Pérez et al., 2007; Pesce et al., 2009; Vera et al., 2010; Villeneuve et al., 2011), except cyanobacteria (Powell et al., 1991). Just like soil bacteria, aqueous microorganisms e.g., microalgae may also utilise glyphosate as source of phosphorous (Wong, 2000). An interesting detail is that glyphosate may be formed during water treatment for purification from organic micropollutants. Glyphosate and AMPA were found to be formed during ozonisation of dilute aqueous solution of the complexing agent ethylenediaminetetra(methylenephosphonic acid) (Klinger et al., 1998; Nowack, 2003). The wide use, and hence ubiquity of glyphosate makes great demands on glyphosate safety, i.e. the absence of any harmful environmental effect except on target organisms (the undesirable weeds).

Glyphosate is very stable in higher plants (Putnam, 1976; Zandstra & Nishimoto, 1977; Chase & Appleby, 1979; Gothrup et al., 1976; Wyrill & Burnside, 1976). Through its metabolism, AMPA has been identified as the main metabolite in plants as well e.g., in montmorency cherry (*Prunus cerasus* L.) leaves, field bindweed (*Convolvulus arvensis* L.), henge bindweed (*Convolvulus sepium* L.), Canada thistle (*Cirsium arvense* (L) Scop.), tall morning glory (*Ipomea purpurea* (L.) Roth.) and wild buckwheat (*Polygonum convolvulus* L.) (Sandberg et al., 1980; Aizawa, 1982; Aizawa, 1989).

Besides AMPA, its certain derivatives e.g., N-methyl-AMPA or N,N-dimethyl-AMPA have been also found as metabolites, mostly in plants (FAO/WHO, 2006). Decomposition in GT plants is even more complex, as some of these plants have been designed for enhanced degradation of glyphosate. In such plants, further AMPA derivatives e.g., N-acetyl-AMPA,

N-malonyl-AMPA, N-glyceryl-AMPA and various conjugates of AMPA have also been identified (FAO/WHO, 2006).

### 5.3 Environmental monitoring of glyphosate

Glyphosate shows unique characteristics in soil as compared to other pesticide active ingredients. With predominantly apolar groups pesticides typically bind to the organic matter in soil (Borggaard & Gimsing, 2008). In contrast, glyphosate is of amphoteric (zwitterionic) character, analytical determination of which is to date a great challenge to analytical chemists. As a result of the unusual chemical behaviour of the parent compound (*N*-phosphonomethylglycine) and its metabolite (AMPA), routine environmental analytical methods do not detect them with sufficient sensitivity. It is also due to the difficult analytical procedure that glyphosate is often not targeted or overlooked in environmental studies, or has been considered of neglectable level. Certain studies, however, report frequent occurrence. In the United States, surface water contamination has been reported due to run-off from agricultural areas (Edwards et al., 1980; Feng et al., 1990) or pesticide drift (Payne et al., 1990; Payne, 1992). Glyphosate has been listed among pesticides of potential concern in surface water contamination in the Mediterranean region of Europe in the mid' nineties (Barceló & Hennion, 1997), and glyphosate and AMPA were found as contaminants in two small tributaries of the river Ruhr in North-Rhine-Westphalia, Germany at up to 590 ng/l concentration (Skark et al., 1998). A monitoring study carried out in Norway found frequent occurrence of glyphosate and its metabolite AMPA in surface water samples. In 54% of the 540 surface water samples collected between 1995 and 1999 glyphosate or AMPA was detected. The maximal concentration was 0.93 µg/l (average 0.13 µg/l) for glyphosate, and 0.2 µg/l (average 0.06 µg/l) for AMPA (Ludvigsen & Lode, 2001a; 2001b). The monitoring study, therefore, indicated broad occurrence of glyphosate and its metabolite at low concentrations. In a study carried out in surface waters of the Midwest in the United States in 2002 glyphosate was detected in 35-40% of the samples (maximal concentration 8.7 µg/l) and AMPA in 53-83% of the samples (maximal concentration 3.6 µg/l) (Battaglin et al., 2005), and both glyphosate and AMPA were detected in vernal snow-flood at concentrations up to 328 and 41 µg/l, respectively, in 2005-2006 in four states of the US (Battaglin et al., 2009). Analysing water samples from 10 wastewater treatment plants in the United States, the U.S. Geological Survey detected AMPA in 67.5% and glyphosate in 17.5% of the samples (Kolpin et al., 2006). The study concluded that urban use of glyphosate contributes to glyphosate and AMPA concentrations in streams in the United States. In a study carried out in Canada in 2004-2005, 21% of the analysed 502 samples contained glyphosate with a maximum concentration of 41 µg/l, and the peak concentration of AMPA was 30 µg/l glyphosate equivalent (Struger et al., 2008). In France, glyphosate and AMPA were detected in 2007 and 2008 due to urban runoff effect (Batta et al., 2009). In fact, Villeneuve et al. (2011) adjudge glyphosate to be one of the herbicides most often found in freshwater ecosystems worldwide, and state that AMPA is the most often detected and glyphosate is the third most frequent pesticide residue in French streams. Elevated glyphosate levels were detected in surface water, soil and sediment samples due to intensive herbicide applications in a GM soybean cultivating area in Argentina (Peruzzo et al., 2008). These studies are warning signs indicating that this herbicide active ingredient of intensive use, that is expected to further expand with the commercial cultivation of GM crops, became an ubiquitous contaminant in surface waters, and therefore, a permanent pollutant factor, which deserves pronounced attention by ecotoxicology.



## 6. Adverse environmental effects of glyphosate

### 6.1 Glyphosate and *Fusarium* species

Sanogo and co-workers (2000) observed that crop loss in soy due to infestation by *Fusarium solani* f. sp. *glycines* increased after glyphosate applications. Kremer and co-workers (2005) described a stimulating effect of the root exudate of GR soy sampled after glyphosate application on the growth of *Fusarium* sp. strains. Treatments caused concentration dependent increase on the mycelium mass of the fungus. Nonetheless, Powel and Swanton (2008) could not confirm these observations in their field study. Kremer and Means (2009) claim that certain fungi utilise glyphosate released from plant roots into the soil as a nutritive, which facilitates their growth. Soil manganese content also affects the above consequence of glyphosate through chelating with the compound and thus, modifying its effects. Considering the fact that numerous plant pathogenic *Fusarium* species produce mycotoxins, an increasing proportion of these species is far not favourable as a side-effect. Johal and Huber (2009) lists numbersome plant pathogens (e.g., *Corynespora cassicola* or *Sclerotinia sclerotiorum* on soy) they claim to grow increasingly after glyphosate treatments, and the list contains several *Fusarium* species (*F. graminearum*, *F. oxysporum*, *F. solani*). They hypothesize that glyphosate causes disturbances in microelement metabolism in plants, and in parallel, deteriorate the defense system of the plants, thereby increasing the virulence of certain plant pathogens. Zobiolo and co-workers (2011) confirmed the above effects by their observation that glyphosate treatments facilitate colonisation of *Fusarium* species on the soy roots, but reduces the fluorescent *Pseudomonas* fraction of the rhizosphere, the level of manganese reducing bacteria and of the indoleacetic acid producing rhizobacteria. As a combined result of these effects, root and overall plant biomasses were found to be reduced.

### 6.2 Toxicity of glyphosate to aquatic ecosystems and amphibians

Substances occurring in surface waters deserve special attention by ecotoxicologists, as they enter a matrix that is the habitat of numerous aqueous organisms and the basis of our drinking water reserves. Drinking water is an irreplaceable essential part of our diet, and is a possible vehicle for chronic exposure (the basis of chronic diseases) in daily contact/consumption.

Glyphosate has been known to cause toxicity to microalgae and other aquatic microorganisms (Goldsborough and Brown 1988; Austin et al., 1991; Anton et al., 1993; Sáenz et al., 1997; DeLorenzo et al., 2001; Ma 2002; Ma et al., 2002; Ma et al., 2003), in fact a green algal toxicity test has been proposed for screening herbicide activity (Ma & Wang, 2002). In contrast, cyanobacteria have been found to show resistance against glyphosate (López-Rodas et al., 2007; Forlani et al., 2008). Tsui and Chu (2003) tested the effect of glyphosate, its most common polyoxyethyleneamine (POEA) type formulating materials, polyethoxylated tallowamines, and the formulated glyphosate preparation (Roundup) on model species from aquatic ecosystems, bacteria (*Vibrio fischeri*), microalgae (*Selenastrum capricornutum*, *Skeletonema costatum*), protozoas (*Tetrahymena pyriformis*, *Euplotes vannus*) and crustaceans (*Ceriodaphnia dubia*, *Acartia tonsa*). The most surprising result of the study was that the assumedly inert detergent formulating agent, POEA was found to be the most toxic component. In light of this it is far not surprising that Cox and Sorgan (2006) and Reuben (2010) propounded the question, why tests only on the active ingredients are necessary to be specified in the documentation required by the Environmental Protection Agency of the

United States (US EPA), when several of the used formulating components are known to exert biological activity.

Although acute toxicity and genotoxicity of glyphosate have been evidenced to certain fish (Langiano & Martinez, 2008; Cavalcante et al., 2008), glyphosate shows favourable acute toxicity parameters on most vertebrates, and therefore, has been classified as III toxicity category by US EPA. The European discretion is stricter, listing the compound among substances causing irritation (Xi) and severe ocular damage (R41). It has to be noted, however, that that model species of neither amphibians, nor reptilians are represented in the toxicological documentations required nowadays. It may not be surprising, therefore, that after atrazine (Hayes et al., 2002; 2010), glyphosate is the second herbicide active ingredient that is questioned due to its detrimental effects on the animal class, considered the most endangered on Earth, amphibians.

Mann and Bidwell (1999) studied the toxicity of glyphosate on tadpoles of four Australian frogs (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis* and *Litoria moorei*). The toxicity of Roundup and its 48-hour LC<sub>50</sub> values were found to be 3-12 mg glyphosate equivalent/l. Tolerance of the adult frogs was substantially greater. A glyphosate-based formulated herbicide preparation (VisionMAX) caused no significant effects on the juvenile adults of the green frogs (*Lithobates clamitans*) when applied at field application doses, only marginal differences in statistics of infection rates and liver somatic indices in relation to exposure estimates (Edge et al., 2011). Chen et al. (2004) observed that the toxicity of glyphosate on the frog species *Rana pipiens* was greatly affected by lacking food resources and the pH of the medium as stress factors. Relyea (2005a) reported tadpole (*Bufo americanus*, *Hyla versicolor*, *Rana sylvatica*, *R. pipiens*, *R. clamitans* and *R. catesbeiana*) mortality related to glyphosate applications. The effect, occurred at 2-16 mg glyphosate equivalent/l concentrations, was linked with the stress caused by the predator of the tadpoles, salamander *Notophthalmus viridescens*. Later Relyea and Jones (2009) included further frog species (*Bufo boreas*, *Pseudacris crucifer*, *Rana cascades*, *R. sylvatica*) into the study, and found LC<sub>50</sub> values to be 0.8-2 mg glyphosate equivalent/l. Testing four salamander species (*Amblystoma gracile*, *A. laterale*, *A. maculatum* and *N. viridescens*), the corresponding values ranged between 2.7 and 3.2 mg glyphosate equivalent/l. In this case, glyphosate was formulated with detergent POEA. Further studies also shed light on the fact that another stress factor, population density, playing an important part in the competition of the tadpoles increased the toxic effect of glyphosate (Jones et al., 2010). Lajmanovich and co-workers (2010) detected lowered enzymatic activities (e.g., acetylcholine esterase and glutathion-S-transferase) in a frog species, *Rhinella arenarum* upon glyphosate treatments.

Sparling and co-workers (2006) detected lowered fecundity of the eggs of the semiaquatic turtle, red-eared slider (*Trachemys scripta elegans*) if treated with glyphosate at high doses.

### 6.3 Teratogenic activity of glyphosate

The teratogenicity of the pesticide preparations containing glyphosate deserves special attention. The very first examples of observed teratogenicity of glyphosate preparations have also been linked to amphibians. Using the so-called FETAX assay, Perkins and co-workers (2000) observed a formulation dependent teratogenic effect of glyphosate on embryos of the frog species *Xenopus laevis*. The concentrations that triggered the effect were relatively high (the highest dose applied in the study was 2.88 mg glyphosate equivalent/l),

but not unrealistically high with respect to field doses of glyphosate, indicating, that high allowed agricultural doses cause glyphosate levels close to the safety margin. Lajmanovich and co-workers (2005) studied the effects of a glyphosate preparation (Glyfos) on the tadpoles of *Scinax nasicus*, and found that a 2-4-day exposure to 3 mg/l glyphosate caused malformation in more than half of the test animals. The treatment was carried out nearly at the LC<sub>50</sub> level of glyphosate. Dallegrove and co-workers (2003) found fetotoxic effects on rats treated with glyphosate at very high, 1000 mg/l concentration on the 6<sup>th</sup>-15<sup>th</sup> day after fertilisation. Nearly half of the newborn rat progeny in the experiments were born with skeletal development disorders.

Testing the effects of glyphosate preparations on the embryos of the sea urchin, *Sphaerechinus granularis*, Marc and co-workers (2004a) observed a collapse of cell cycle control. Inhibition affects DNA synthesis in the G2/M phase of the first cell cycle (Marc et al., 2004b). The authors estimate that glyphosate production workers inhale 500-5000-fold level of the effective concentration in these experiments. A marked toxicity of the formulating agent POEA has also been observed on sea urchins (Marc et al., 2005). The very early DNA damage was claimed to be related to tumour formation by Bellé and co-workers (2007), and the authors consider the sea urchin biotest they developed as a possible experimental model for testing this effect. Jayawardena and co-workers (2010) described nearly 60% developmental disorders on the tadpoles of a Sri Lanka frog (*Polypedates cruciger*) upon treatment with 1 ppm glyphosate.

The teratogenicity of herbicides of glyphosate as active ingredient have been tested lately on amphibian (*X. laevis*) and bird (*Gallus domesticus*) embryos. Applied with direct injection at sublethal doses caused modification of the position and pattern of rhombomeres, the area of the neural crest decreased, the anterior-posterior axis shortened and the occurrence of cephalic markers was inhibited at the embryonic development stage of the nervous system. As a result, frog embryos became of characteristic phenotype: the trunk is shortened, head size is reduced, eyes were improperly or not developed (microphthalmia), and additional cranial deformities occurred in later development. Similar teratogenic effects were seen on embryos of Amniotes e.g., chicken. These developmental disorders may be related to damages of the retinoic acid signal pathway, resulting in the inhibition of the expression of certain essential genes (*shh*, *slug*, *otx2*). These genes play crucial roles in the neurulation process of embryogenesis (Paganelli et al., 2010). These findings were later debated by several comments. On behalf of the producers, Saltmiras and co-workers (2011) questioned certain conclusions in the work of Paganelli and co-workers (2010), claiming that the standardised pilot teratogenicity tests, carried out under good laboratory practice (GLP) by the manufacturers, have been evaluated by independent experts of several international organisations. They also considered the dosages used by Paganelli and co-workers exceedingly high, and the mode of application (microinjection) unrealistic in nature. Similar criticism has been voiced by Mulet (2011) and Palma (2011). In his answer, Carrasco (2011) emphasised their opinion that the company representatives ignore scientific facts supporting teratogenicity of atrazine, glyphosate and triadimefon through retinoic acid biosynthesis. He also emphasized that of 180 research reports of Monsanto, 150 are not public, or have never been presented to the scientific community. He also included that they obtained similar phenotypes in their studies with microinjection, than by incubation of the preparations. As a follow-up, Antoniou and co-workers (2011) compiled an extensive review of 359 studies and publications on the teratogenicity and birth defects caused by glyphosate,

and heavily criticize the European Union for not banning glyphosate, but rather postponing its re-evaluation until 2015 (European Commission, 2010).

#### 6.4 Genotoxicity of glyphosate

Occupational exposure to pesticides, including glyphosate as active ingredient, may lead to pregnancy problems even through exposure of men (Savitz et al., 1997). Such phenomenon has been first described in epidemiology with Vietnam War veterans exposed to Agent Orange with phenoxyacetic acid type active ingredients contaminated with dibenzodioxins. Although glyphosate has been claimed not to be genotoxic and its formulation Roundup “causing only a week effect” (Rank et al., 1993; Bolognesi et al., 1997), Kale and co-workers (1995) observed mutagenic effects of Roundup in *Drosophila melanogaster* recessive lethal mutation tests. Lioi and co-workers (1998) described increasing sister chromatid exchange in human lymphocytes with increasing glyphosate doses. Walsh and co-workers (2000) detected in murine tumour cells the inhibitory activity of Roundup on the biosynthesis of a protein (StAR) participating in the synthesis of sex steroids. This reduced the operation of the cholesterol - pregnenolon - progesteron transformation pathway to a minimal level. As it often happens in exploring mutagenic effects of chemical substances, additional studies have not found glyphosate mutagenic, and therefore, it is not so listed in the GAP2000 program compiled from US EPA/IARC databases. However, Cox (2004) describes chronic toxicity profile of several substances applied in the formulation of glyphosate.

Studying the activity of dehydrogenase enzymes in the liver, heart and brain of pregnant rats, Daruich and co-workers (2001) concluded that glyphosate causes various disorders both in the parent female and in the progeny. According to results of the study by Benedettia and co-workers (2004), aminotransferase enzyme activity decreased in the liver of rats, impairing lymphocytes, and leading to liver tissue damages. In *in vitro* tests McComb and co-workers (2008) found that glyphosate acts in the mitochondria of the rat liver cells as an oxidative phosphorylation decoupling agent. Mariana and co-workers (2009) observed oxidative stress status decay in the blood, liver and testicles upon injection administration of glyphosate, possibly linked to reproductional toxicity.

Prasad and co-workers (2009) detected cytotoxic effects, as well as chromosomal disorders and micronucleus formation in murine bone-marrow. Poletta and co-workers (2009) described genotoxic effects of Roundup on the erythrocytes in the blood of caimans, correlated with DNA damages.

According to the survey of De Roos and co-workers (2003), the risk of the incidence of non-Hodgkin lymphoma is increased among pesticide users. As the authors found it, this applies to herbicide preparations with glyphosate as active ingredient. Focusing the study solely on glyphosate preparations a year later in the corn belt of the United States, of the majority of malignant diseases, only the incidence of abnormal plasma cell proliferation (*myeloma multiplex*, *plasmocytoma*) showed a slight rise (De Roos et al., 2004). Myeloma represents approximately 10% of the malignant haematological disorders. Although the cause of the disease is not yet known, its risk factors include autoimmune diseases, certain viruses (*HIV* and *Herpes*), and the frequent use of certain solvents as occupational hazard. On the basis of murine skin carcinogenesis, George and co-workers (2010) reported that glyphosate may act as a skin tumour promoter due to the induction of several special proteins.

### 6.5 Hormone modulant effects of glyphosate and POEA

Studying chronic exposure of tadpoles of *Rana pipiens*, Howe and co-workers (2004) found that in addition to developmental disorders, gonads in 15-20% of the treated animals developed erroneously, and these animals showed intersexual characteristics. Arbuckle and co-workers (2001) registered increased risk of abortion in agricultural farms after glyphosate applications. In addition, excretion of glyphosate has been determined in the urine of agricultural workers and their family members (Acquavella et al., 2004).

Richard and co-workers (2005) evidenced toxicity of glyphosate on the JEG3 cells in the placenta. Formulated Roundup exerted stronger effect than glyphosate itself. Glyphosate inhibited aromatase enzymes of key importance in estrogen biosynthesis. This effect has also been evidenced in *in vitro* tests by binding to the active site of the purified enzyme. The formulating agent in the preparation enhanced the inhibitory effect in the microsomal fraction. Benachour and co-workers (2007) tested the effect of glyphosate and Roundup Bioforce on various cell lines, and also determined the aromatase inhibiting effect of glyphosate and the synergistic effect of the formulating agent. They suppose that the hormone modulant effect of Roundup may affect human reproduction and fetal development. Testing these human cell lines, Benachour and Séralini (2009) found that glyphosate alone induces apoptosis, and POEA and AMPA applied in combination exert synergistic effects, similarly to the synergy seen for Roundup. The synergy was reported to be further acerbated with activated Cry1Ab toxin related to that produced by insect resistant GM plants, raising concern regarding stacked genetic event GM crops exerting both glyphosate tolerance and Cry1Ab based insect resistance (Mesnage et al., 2011). Moreover, the combined effect caused cell necrosis as well. Effect enhancement is likely to be explained by the detergent activity of POEA facilitating the penetration of glyphosate through cell membranes and subsequent accumulation in the cells. The aromatase inhibitory effect of the formulated preparation was four-fold, as compared to the neat active ingredient. The authors consider it proven, that POEA, previously believed to be inert, is far not inactive biologically. As the authorised MRL of glyphosate in forage is as high as 400 mg/kg, Gasnier and co-workers (2009) studied in various *in vitro* tests, what effects this may cause in a human hepatic cell line. All treatments indicated a concentration-dependent effect in the toxicity tests were found genotoxic in the comet assay for DNA damages, moreover, displayed antiestrogenic and antiandrogenic effects.

### 6.6 Glyphosate resistance of weeds

Frequent applications of glyphosate and the spread of GT crops outside of Europe escalate the occurrence of glyphosate in the environment, exerting severe selection pressure on the weed species. It has been well known that certain weeds have native resistance against glyphosate e.g., the common lambsquarters (*Chenopodium album*), the velvetleaf (*Abutilon theophrasti*) and the common cocklebur (*Xanthium strumarium*).

The first population of GT *Lolium rigidum* was described in 1996 by Pratley and co-workers in Australia. This was followed in 1997 by GT goosegrass (*Eleusine indica*) in Malaysia (Lee & Ngim, 2000), GT horseweed (*Conyza canadensis*) in the United States (VanGessel, 2001), GT Italian ryegrass (*Lolium multiflorum*) in Chile (Perez & Kogan, 2003). Further known GT weed species include *Echinochloa colona* (2007), *Urochloa panicoides* (2008) and *Chloris truncata*

(2010) in Australia; *Conyza bonariensis* (2003) and ribwort plantain (*Plantago lanceolata*, 2003) in South Africa; ragweed (*Ambrosia artemisiifolia*, 2004), *Ambrosia trifida* (2004), *Amaranthus palmeri* (2005), *Amaranthus tuberculatus* (2005), summer cypress (*Bassia scoparia*, 2007) and annual meadow grass (*Poa annua*, 2010) in the United States; *Conyza sumatrensis* (2009) in Spain; Johnsongrass (*Sorghum halepense*) (2005), Italian ryegrass (*Lolium perene*, 2008) in Argentina; *Euphorbia heterophylla* (2006) in Brazil; *Parthenium hysterophorus* (2004) in Colombia and *Digitaria insularis* (2006) in Paraguay (Heap, Epubl). GT Johnsongrass was reported in a continuous soybean field in Arkansas, United States (Riar et al., 2011). Price (2011) claims that agricultural conservation tillage is threatened in the United States by the rapid spread of GT Palmer amaranth (*Amaranthus palmeri* [S.] Wats.) due to wide range cultivation of transgenic, GT cultivars and corresponding broad use of glyphosate. GT amaranths were first identified in Georgia, and later reported in nine states, Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee, and a closely related GT amaranth, common waterhemp (*Amaranthus rudis* Sauer) in four states, Illinois, Iowa, Minnesota, and Missouri. Moreover, GT Italian ryegrass populations collected in Oregon, United States appeared to show cross-resistance to another phosphonic acid type herbicide active ingredient, glufosinate (Avila-Garcia & Mallory-Smith, 2011).

Powles and co-workers (1998) described a *L. rigidum* population resisting 7-11-fold dosage of glyphosate in Australia. Shrestha and Hemree (2007) found GT subpopulations of 5-8 leaf stage *Conyza canadensis* surviving only 2-4-fold glyphosate doses. According to Powles (2008), it is not coincidental that in countries, where GT crops are on the rise (Argentina and Brazil), the occurrence of GT weeds is more frequent. Moreover, he considers this one of the main obstacles of the spread of GT crops in the agricultural practice. Glyphosate tolerance is an inherited property, therefore, accumulation of weeds in the treated areas is to be expected. Genomics studies of the GT populations revealed that mutation of the gene (*epsps*) encoding the target enzyme responsible for tolerance is not infrequent in nature. (The mutant alleles (*mepsps*, *2mepsps*) responsible for tolerance has been found in maize as well, see Table 2.). Reduced or modified uptake or translocation of glyphosate has also been observed, and the metabolic fate of the compound may also become altered in the cell (Shaner, 2009), possibly resulting in GT populations. It is not difficult to predict, that prolonged cultivation of GT crops will necessitate supplemental herbicide administrations with active ingredients other than glyphosate.

## 7. References

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