Acute Toxicity of Glyphosate-Based Formulations to Five Aquatic Organisms of Different Trophic Levels

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Abstract Glyphosate (N-phosphonomethyl glycine) and its formulations, such as Roundup®, are the most widely used herbicides all over the world. A number of glyphosate-based formulations containing the surfactant Polyethoxylated tallow amine (POEA) at different ratios are sold for the same purposes. Recently, there is a big debate about Glyphosate safety and the presence of POEA in the herbicide formulations has raised concerns regarding its toxicity to mammals, invertebrates and wildlife. The current study was undertaken to provide data on the acute toxicity of two glyphosate-based formulations (namely: Herfosate 36%EC and Glyphoid 48%EC) compared with Glyphosate (95%WP), using five aquatic organisms representing different trophic levels. Chemical analysis revealed presence of POEA in Herfosate and Glyphoid formulations, while the parent Glyphosate was free of POEA. The most toxic herbicide to the five tested organisms was Glyphoid which contained the highest amount of POEA, followed by Herfosate, and then Glyphosate. Based on the obtained LC_{50} values, the sensitivity of the tested organisms to the three herbicides could be arranged in the following order: **i) Herfosate:** *D. magna > C. pipiens > V. fischeri > B. alexandrina > G. affinis,* **ii) Glyphoid:** *D. magna > C. pipiens > V. fischeri > G. affinis > B. alexandrina,* and **iii) Glyphosate:** *D. magna > V.* fischeri *> C. pipiens > B. alexandrina > G. affinis.* The present study provides further data that may help pesticide regulatory agencies to undertake proper management towards regulation of glyphosate-based formulations.

Keywords Glyphosate-based formulations, Acute toxicity, Aquatic organisms, Polyethoxylated tallow amine (POEA)

Introduction

Glyphosate [N-(phosphonomethyl) glycine] or 2-[(phosphonomethyl) amino] acetic acid, ($C_3H_8NO_5P$), is a broad spectrum, non-selective, systemic, post-emergence herbicide. It has been used extensively throughout the world over the past three decades. Glyphosate is the active ingredient produced by Monsanto in 1974 under the trade name Roundup® [1]. Glyphosate-based formulations are the commonly used products all over the world. About fifty formulations of different trade names are registered in Egypt for the year 2017. Only two are potassium salts and the rest are ammonium salts in which Glyphosate comprises 24 - 75% as active ingredient (a.i.), but the majority of formulations contain 48% a.i. The herbicide is a powerful weed killer for annual and perennial herbs in crop and non-crop lands. The surfactant Polyethoxylated tallow amine (POEA) refers to a range of non-ionic substances used as emulsifiers and wetting agents for formulation of agrochemical, such as pesticides and herbicides (CAS No. 61791-26-2), the U.S. EPA List 3 of Inert Ingredients of Pesticides).

On March 20, 2015, the International Agency for Research on Cancer (IARC), an institution of the World Health Organization (WHO), has classified glyphosate as "probably carcinogenic to humans (Group 2A). The complete monograph on glyphosate (Volume 112) was published on 29 July 2015 [2]. Since that time, there is a big debate about Glyphosate safety. The European Food Safety Authority (EFSA) claims that there is

insufficient scientific evidence of a cancer link [3]. The Joint Meeting of FAO/WHO on Pesticide Residues declared that Glyphosate is "unlikely to pose a carcinogenic risk to humans from exposure through the diet" [4]. *Additives or Co-formulants* are used to produce pesticides, but are neither active substances, safeners, nor synergists. They can be banned or restricted for use at national level. The surfactant POEA, one of the *co-formulants* used for glyphosate-based products, has raised concerns regarding its toxicity to mammals, invertebrates and wildlife [5]. The EU Commission has proposed to Member States to ban POEA as a co-formulant in glyphosate-based products. A final decision is expected to be run out on 31 December 2017.

In front of such critical situation, scientists are being harried to provide scientific evidences with respect to human carcinogenicity and risks to wildlife (e.g., aquatic organisms) of glyphosate - based formulations. From this stand point, the present investigation was undertaken to introduce acute toxicity data on Glyphosate (95% WP) and two Glyphosate-based formulations coined as "Herfosate-36%EC" and "Glyphoid-48%EC" against five different aquatic organisms, namely: the microcrustacean (*Daphnia magna*), the mosquito larvae (*Culex pipiens*), the mosquito fish (*Gambusia affinis*), the Bilharzias snail (*Biomphalaria alexandrina*), and the marine water bacteria (*Vibrio fischeri*). While the obtained results will have their importance to local regulations, they may provide useful data to the Glyphosate File waiting for global action by the end of 2017.

Material and Method
Test herbicides
Formulated isopropylamine salt of Glyphosate, N-phosphonomethyl glycine, $C_3H_8NO_5P$, as (95%WP) and two glyphosate-based formulations; namely Herfosate (36% EC) and Glyphoid (48% EC) were obtained from Pesticide Residue Analysis Laboratory, Ministry of Agriculture and Land Reclamation, Egypt. In the course of the lab duties in checking the presence of the surfactant **POEA** in such formulations, Glyphosate proved to be free of **POEA**. The latter additive comprised 15% in Glyphoid and 10% in Herfosate formulations.

Test organisms
Five aquatic organisms namely: *Daphnia magna* (microcrustacea) , *Culex pipiens* larvae (mosquito), Gambusia affinis (fish), *Biomphalaria alexandrina* (snail), and *Vibrio fischeri* (bacteria). The latter organism is ready prepared commercially in a form of dry powder and purchased from Modern Water Inc., New Castle, DE 19720, USA; specifically for "Microtox$^®$ test". The other four organisms are reared in the Environmental Toxicology Research Unit (ETRU), National Research Centre, Cairo, Egypt, according to standard specifications for each organism.

In brief, adult females of *C. pipiens* mosquito were fed on pigeons trapped in cages containing small plastic containers filled with distilled water and a small amount of tetramine [6]. The eggs laid were watched for emerging the 1st instar larvae used in bioassay.

Bulk cultures of *Daphnia magna* maintained in our laboratory were transferred into glass beakers containing ASTM hard synthetic water [7] and suspension of *Scenedesmus subspicatus* for animal feeding [8]. The culture medium was changed every other day and neonates (<24 h) were collected for toxicity studies.

The fish, *Gambusia affinis* are cultured in large glass aquaria containing well dechlorinated tap water and connected to aerating pumps. The fishes were fed on commercial pelleted diet once a day. Individuals of approximately 0.5-1.0 gm body weight and 2.0-3.0 cm body length were transferred to an aquarium just before running the tests.

The snails, *Biomphalaria alexandrina* were originally obtained from Theodore Bilharz Research Institute, Imbaba, Giza. They are reared along with the same *Gambusia* fish aquaria in the laboratory and fed green lettuce leaves. Adult snails of 8-10 mm shell diameter were transferred to an aquarium just before running the tests.

Test procedures
Stock solutions of the tested herbicides were prepared in distilled or deionized water. Preliminary tests were conducted to select proper dilutions for each tested organism. At least four concentrations prepared on active ingredient (a.i.) contents were used and each concentration was repeated three times and kept at room

temperature for 48h, except in the Microtox tests in which exposure time was 15min only. Control tests were carried out along with the other treatments but in water free of the herbicides. After the specified duration times, percent kill was computed and adjusted by Abbott's formula [9]. Probit analysis [10] was applied to construct LC-P lines for estimating toxicity data (e.g., LC_{50}, fudicial limits and slopes) for each herbicide against each tested organism.

Except the Microtox test, other tests were carried out according to the standard test methods (e.g., Write [11] for mosquitoes; OECD [12] for daphnids, WHO [13] for snails; and US-EPA [14] for fish). For convenience, details on Microtox test is given below.

The freeze-dried luminescent bacteria, *Vibrio fischeri* (13F4067A), reconstitution solution (AFZ686016), Osmotic Adjusting Solution (20% NaCl; AFZ686019), and diluent solution (2% NaCl; AFZ686011) were supplied by Modern Water Inc., New Castle, DE 19720, USA. The tests were performed using the Microtox® Model 500 Toxicity Analyzer from Modern Water Inc. The analyzer was equipped with a 30-well temperature-controlled incubator chamber at 15°C. A small compartment held at 5 °C was used to store the bacteria before dilution. The light output was recorded from a digital display.

Based on active ingredient (a.i.) content in each of the tested herbicide, the test solutions were prepared in deionized tap water. Preliminary experiments were carried out in order to find out the most suitable concentration range allowing the determination of the EC_{50} (or LC_{50}) values. The solutions were freshly prepared and adjusted to pH 6.0 by addition of 0.1N-HCl solutions and used immediately. Each assay was performed at least in triplicate.

EC_{50} values, defined as the concentration which provokes a 50% light reduction on *V. fischeri*, were obtained by following the Microtox® basic test protocol [15]. According to Finney [10], the 15 min-EC_{50} values were estimated by regression analysis of the linear relationship between the logarithms of the toxicant concentration against the logarithm of the lost/remaining light intensity ratio "gamma".

Results

At test concentrations which were selected to give a reasonable scale of mortality ranging between ca. 20% and ca. 90% mortality, the three used herbicides were bioassayed against the five different organisms.

Table 1 presents concentration-mortality data for the three tested herbicides on the 1st instars' mosquito larvae of *Culex pipiens* (*C.pipiens*). Generally, mortalities in larvae showed gradual increases with the increase of the tested concentrations. For example, Herfosate at concentrations of 60, 250, 400 and 600 ppm caused mortalities accounted to 20.0, 55.0, 72.5 and 85.0%, respectively. While similar mortalities were nearly obtained with very higher concentrations from Glyphosate.

Table 1: Concentration-mortality relationship for glyphosate-based formulations compared with glyphosate in tests against mosquito *Culex pipiens* larvae

Herfosate (36% EC)		Glyphoid (48% EC)		Glyphosate (95% WP)	
Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)
60	20.0	40	23.1	2000	25.0
250	55.0	60	43.5	3000	42.5
400	72.5	100	69.6	5000	59.0
600	85.0	200	81.5	7000	84.6

* Exposure time: 48 h.

Toxicity results on *Daphnia magna* (*D. magna*) neonates are presented in table 2. The herbicide Glyphoid caused 23.3, 40.6, 70.0 and 77.4% mortalities at concentrations of 20, 40, 60 and 80 ppm, respectively. In comparison, such values for the herbicide Herfosate were 33.3, 63.3, 83.8 and 90.6 % mortalities at 60, 250, 400 and 600 ppm concentrations. Nearly, similar mortality results were achieved by very higher concentrations from the herbicide Glyphosate.

Table 2: Concentration-mortality relationship for glyphosate-based formulations compared with glyphosate in tests against *Daphnia magna* neonates

Herfosate (36% EC)		Glyphoid (48% EC)		Glyphosate (95% WP)	
Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)
60	33.3	20	23.3	600	26.2
250	63.3	40	40.6	800	52.5
400	83.8	60	70.0	1000	78.9
600	90.6	80	77.4	1300	84.6

* Exposure time: 48 h.

The bioassay results for the tested herbicides against the freshwater snail, *Biomphalaria alexandrina* (*B. alexandrina*) are shown in table 3. The highest mortality (87.5%) was obtained at 800 ppm of Herfosate, while that for Glyphoid (88.9%) was obtained at 700 ppm. To get mortalities ranging from 23.5% to 83.3%, concentrations of Glyphosate were found to be 3000 ppm to 7000 ppm.

Table 3: Concentration-mortality relationship for glyphosate-based formulations compared with glyphosate in tests against *Biomphalaria alexandrina* snails

Herfosate (36% EC)		Glyphoid (48% EC)		Glyphosate (95% WP)	
Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)
200	20.8	150	25.0	3000	26.9
400	41.6	300	45.8	4000	33.3
600	68.0	500	70.8	6000	65.4
800	87.5	700	88.0	7000	80.8

* Exposure time: 48 h.

Generally, to get data required to establish lethal concentration-mortality lines (LC-P lines) for the tested herbicides against the fish, *Gambusia affinis* (*G. affinis*), it was necessary to prepare a series of concentrations ranging from 12000 -18000 ppm for Herfosate; 120-700 ppm for Glyphoid; and 20000-50000 ppm for Glyphosate (Table 4).

Table 4: Concentration-mortality relationship for glyphosate-based formulations compared with glyphosate in tests against *Gambusia affinis* fish

Herfosate (36% EC)		Glyphoid (48% EC)		Glyphosate (95% WP)	
Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)
12000	22.2	120	27.8	20000	23.5
14000	44.5	300	50.0	30000	44.4
16000	55.6	500	72.2	40000	64.7
18000	77.8	700	88.9	50000	83.3

* Exposure time: 48 h.

The situation was different with the bioassay of the marine bacterium, *Vibrio fischeri* (*V. fischeri*) (Microtox® test). According to the data presented in table 5, a 85% mortality (i.e., inhibition) in the bacteria was reached at concentrations equaled to 870, 550 and 1430 ppm , respectively from Herfosate, Glyphoid and Glyphosate.

Table 5: Concentration-inhibition relationship for glyphosate-based formulations compared with glyphosate in tests against *Vibrio fischeri* bacteria (Microtox®)

Herfosate (36% EC)		Glyphoid (48% EC)		Glyphosate (95% WP)	
Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)	Concentration (ppm)	Mortality (%)
47	20.3	22	11.1	570	20.0
120	32.2	41	16.1	770	32.3
300	62.5	75	35.7	1040	66.7
870	85.0	550	85.0	1430	85.0

* Exposure time: 15 min.

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The tabulated data (Tables 1-5) were used to estimate the lethal concentrations for 50% in the tested organisms (LC_{50} values or IC_{50} values in Microtox® test). Such LC_{50} or IC_{50} values represent the toxicity value of any tested substance against a given organism. Table 6 presents toxicity data for the tested herbicides against the five aquatic organisms. It was cleared that the most toxic herbicide to the five tested organisms was Glyphoid followed by Herfosate, and then Glyphosate.

Table 6: Collective toxicity data for the tested herbicides against five aquatic organisms

Test Organism	Parameter	Herbicides		
		Herfosate (36% EC)	Glyphoid (48% EC)	Glyphosate (95% WP)
C. pipiens larvae	LC50	186.1	72.7	3561.9
	95% CL	(150 -220)	(64 - 83)	(3180 - 4000)
	Slope	1.8	2.4	2.9
D. magna neonates	LC50	118.5	41.6	773.1
	95% CL	(90 -150)	(37- 47)	(720 - 820)
	Slope	1.7	2.6	5.3
B. alexandrina snails	LC50	404.7	393.5	4618.7
	95% CL	(360 - 450)	(360 - 430)	(4270 - 4980)
	Slope	3.1	2.7	4.1
G. affinis fish	LC50	14180	240	31134
	95% CL	(14029-15410)	(137- 329)	(28750 - 33510)
	Slope	1.7	10.1	4.1
V. fischeri bacteria	LC50	201	132.8	883.7
	95% CL	(160 - 250)	(110 - 170)	(830 - 940)
	Slope	1.5	1.7	4.9

N.B.:

- LC50 = IC50 for Microtox test (*V.fischeri* bacteria).
- CL: confidence limits.
- Exposure time in Microtox test = 15 min.
- Exposure time for the rest organisms = 48h.

The sensitivity of the tested organisms to the three herbicides could be arranged in the following order:

i)   **Herfosate:** *D. magna > C. pipiens > V. fischeri > B. alexandrina > G. affinis*
ii)   **Glyphoid:** *D. magna > C. pipiens > V. fischeri > G. affinis > B. alexandrina*
iii)   **Glyphosate:** *D. magna > V. fischeri > C. pipiens > B. alexandrina > G. affinis*

Since *D. magna* was the most sensitive among the other organisms towards the tested herbicides, the LC_{50} in *Daphnia* (Table 6) could be considered as a base for calculating the "relative potency". According to the data shown in table 7, the potency of *C. pipiens, B. alexandrina, G. affinis and V. fischeri* relative to *D. magna* equaled 63.7, 29.3, 0.84 and 59.0%, respectively for Herfosate herbicide. For Glyphoid and Glyphosate herbicides, the relative potency values were completely different according to the tested herbicide.

Table 7: Relative potency of the bioassayed herbicides against the tested organisms.

Herbicide	*Relative Potency (%) / Tested Organisms				
	D. magna	*C. pipiens*	*B. alexandrina*	*G. affinis*	*V. fischeri*
Herfosate	100.0	63.7	29.3	0.84	59.0
Glyphoid	100.0	57.2	10.6	17.3	31.3
Glyphosate	100.0	21.7	16.7	2.5	87.5

*Relative Potency = LC_{50} of *Daphnia* divided by LC_{50} of a test in question multiplied by 100.

LC_{50} values refer to Table 6.

Discussion

The active ingredient of pesticides is combined with other substances to create the commercial formulated product on the market. These additives or adjuvants include a wide array of compounds. Information regarding

some of these additives is considered confidential and not free-access for the public. The toxic effects of the pesticide may be a consequence of the active ingredient or other additives in the formulation or both [16].

The herbicide glyphosate is not applied in the field as a pure active ingredient, but sold worldwide under a variety of commercial products. Therefore, the toxicity of commercial products should be assayed [17]. Previous studies revealed that glyphosate formulations are more toxic than the active ingredient itself; supporting the role of additives in increasing toxicity of the commercial formulations [18, 19].

Several investigators have reported higher toxicity of glyphosate-based formulations compared with the active ingredient due to the surfactant polyoxyethylene amine, (POEA), which is added to several glyphosate formulations [20-23]. In toxicological assessments included the isopropylamine (IPA) salt of glyphosate compared with glyphosate-POEA formulations (e.g., Roundup®) or the surfactant POEA, the toxicity of the IPA-glyphosate was the lowest in the majority of tests. Such findings were reported either towards mammalian organisms [17-18, 20] or fishes and aquatic invertebrate organisms [21, 23-27].

Half-lives of glyphosate and its main breakdown product, aminomethylphosphonic acid (AMPA), in soil range from 2 to 197 days and 76 to 240 days, respectively. They can persist as residues in soils and crops for up to 3 years, and could be leached into reservoirs and drainage canals [28]. Because fish and aquatic invertebrates occur in reservoirs and connecting canals in which several glyphosate-based formulations may be applied, it is essential to determine whether such herbicides affect non-target aquatic organisms, starting from laboratory bioassay. The present study includes acute toxicity evaluation for two glyphosate-based formulations compared with the isopropylamine (IPA) salt of glyphosate against five aquatic organisms of different trophic levels; one of them is a marine bacterium (*V. fischeri*) and the other four organisms are candidates of freshwater organisms.

Several investigators have tested toxicity of different glyphosate formulations against different aquatic organisms including *Vibrio fischeri* (Microtox® bacterium) and the crustaceans (*Ceriodaphnia dubia)*. Generally, the toxicity order of the chemicals was: POEA > Roundup® > glyphosate acid > IPA salt of glyphosate [25]. This means that the surfactant (POEA) was the most toxic followed by Roundup® which contains POEA. Similar studies were also conducted on *Daphnia magna* by Folmar et al. [24] and Cuhra [26] who reported that low levels of glyphosate-based formulations induce significant negative effects on this important microcrustacean. Cuhra [26] suggested the need to revise the current toxicity classification of these chemicals to aquatic invertebrates.

In the current study, the toxicity order of the bioassayed herbicidal formulations against the tested organisms were: Glyphoid > Herfosate > IPA salt of glyphosate. Taking into consideration that Glyphoid formulation has proved to contain 15% of POEA, corresponding to 10% in Herfosate, and none in IPA formulation, this leads us to suggest that the presence of POEA in glyphosate-based formulations affected the toxicity of these preparations. Such findings are supported by the previously reported about the role of the surfactant , POEA, in glyphosate-based formulation's toxicity [20-23, 25].

The literature offers much information about toxicity of glyphosate and its formulations on different fish species, such as *Oncorhynchus mykiss, Oncorhynchus nerka, Cyprinus carpio, and others* . To the best of our knowledge, there is no previous studies on the mosquitofish, *Gambusia affinis.* G. *affinis* is a biological predator to aquatic stages of mosquitoes; therefore, both organisms were included in the present study. The findings of the current investigation (Table 6) indicate big differences between LC_{50} values estimated to both the fish and the mosquito; results may suggest "selective toxicity" in favor of the fish. These findings are supported by our previous study in which the potential voracity of *G. affinis* on *C. pipiens* larvae was elucidated [29].

In this respect, it may be useful to mention that laboratory toxicity data contrasting responses of aquatic organisms to pesticides are important for focusing on sensitive species and their homogeneity to a pesticide in question; based on the steepness of exposure-response curves (i.e., the slope values). These data also allow prediction of expected responses of aquatic species to a range of concentrations *in situ* [30]. *For instance, the slope value of regression line in the bioassay of Glyphoid against G. affinis equaled (10.1), compared with 1.7 and 4.1, respectively for Herfosate and Glyphosate (Table 6). Such results indicate to how much the fish responded differentially to the tested herbicides.*

The 48h-LC$_{50}$ of Roundup® herbicide against the mollusc, *Utterbackia imbecillis* was found 18.3 ppm [31]. In comparison, Herfosate and Glyphoid, which are forms of Roundup®, have shown LC$_{50}$ values of 404.7 and 393.5 ppm, respectively against *Biomphalaria alexandrina* snail (Table 6); a result which indicate the higher sensitivity of *U. imbecillis* to the herbicide Roundup®.

Conclusion

The acute toxicity of the tested glyphosate preparations in this study against five different aquatic organisms revealed the link between the content of POEA in glyphosate-based formulations and their toxicity to the tested organisms. The toxicity order of the bioassayed herbicidal formulations against the tested organisms were: Glyphoid > Herfosate > IPA salt of glyphosate. In all cases, the microcustacean, *D. magna* was the most sensitive organism, while *G. affinis* fish was the lowest sensitive in Herfosate and glyphosate tests and *B. alexandrina* snail in Glyphoid tests. The findings of the present study provide further data that may help pesticide regulatory agencies to undertake further management towards regulation of glyphosate-based formulations.

Conflict of interest: The authors declare that there is no conflict of interest.

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